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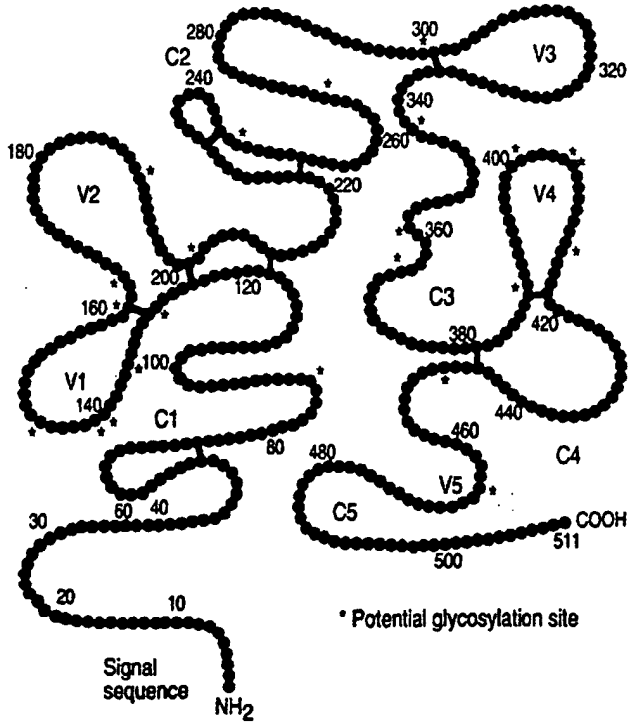
(54) Title: HIV ENVELOPE POLYPEPTIDES

(57) Abstract

A method for the rational design and preparation of vaccines based on HIV envelope polypeptides is described. In one embodiment, the method for making an HIV gp120 subunit vaccine for a geographic region comprises determining neutralizing epitopes in the V2 and/or C4 domains of gp120 of HIV as depicted in the figure. In a preferred embodiment of the method, neutralizing epitopes for the V2, V3 and C4 domains of gp120 are determined. Also described are DNA sequences encoding gp120 from preferred vaccine strains of HIV.



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HIV ENVELOPE POLYPEPTIDES

FIELD OF THE INVENTION

This invention relates to the rational design and preparation of HIV vaccines based on HIV envelope polypeptides and the resultant vaccines. This invention further relates to improved methods for HIV serotyping and immunogens which induce antibodies useful in the serotyping methods.

BACKGROUND OF THE INVENTION

Acquired immunodeficiency syndrome (AIDS) is caused by a retrovirus identified as the human immunodeficiency virus (HIV). There have been intense effort to develop a vaccine. These efforts have focused on inducing antibodies to the HIV envelope protein. Recent efforts have used subunit vaccines where an HIV protein, rather than attenuated or killed virus, is used as the immunogen in the vaccine for safety reasons. Subunit vaccines generally include gp120, the portion of the HIV envelope protein which is on the surface of the virus.

The HIV envelope protein has been extensively described, and the amino acid and RNA sequences encoding HIV envelope from a number of HIV strains are known (Myers, G. et al., 1992. Human Retroviruses and AIDS. A compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, New Mexico). The HIV envelope protein is a glycoprotein of about 160 kd (gp160) which is anchored in the membrane bilayer at its carboxyl terminal region. The N-terminal segment, gp120, protrudes into the aqueous environment surrounding the virion and the C-terminal segment, gp41, spans the membrane. Via a host-cell mediated process, gp160 is cleaved to form

gp120 and the integral membrane protein gp41. As there is no covalent attachment between gp120 and gp41, free gp120 is released from the surface of virions and infected cells.

The gp120 molecule consists of a polypeptide core of 60,000 daltons which is extensively modified by N-linked glycosylation to increase the apparent molecular weight of the molecule to 120,000 daltons. The amino acid sequence of gp120 contains five relatively conserved domains interspersed with five hypervariable domains. The positions of the 18 cysteine residues in the gp120 primary sequence, and the positions of 13 of the approximately 24 N-linked glycosylation sites in the gp120 sequence are common to all gp120 sequences. The hypervariable domains contain extensive amino acid substitutions, insertions and deletions. Sequence variations in these domains result in up to 30% overall sequence variability between gp120 molecules from the various viral isolates. Despite this variation, all gp120 sequences preserve the virus's ability to bind to the viral receptor CD4 and to interact with gp41 to induce fusion of the viral and host cell membranes.

gp120 has been the object of intensive investigation as a vaccine candidate for subunit vaccines, as the viral protein which is most likely to be accessible to immune attack. gp120 is considered to be a good candidate for a subunit vaccine, because (i) gp120 is known to possess the CD4 binding domain by which HIV attaches to its target cells, (ii) HIV infectivity can be neutralized *in vitro* by antibodies to gp 120, (iii) the majority of the *in vitro* neutralizing activity present in the serum of HIV infected individuals can be removed with a gp120 affinity column, and (iv) the gp120/gp41 complex

appears to be essential for the transmission of HIV by cell-to-cell fusion.

The identification of epitopes recognized by virus neutralizing antibodies is critical for the rational design of vaccines effective against HIV-1 infection. One way in which antibodies would be expected to neutralize HIV-1 infection is by blocking the binding of the HIV-1 envelope glycoprotein, gp120, to its cellular receptor, CD4. However, it has been surprising that the CD4 blocking activity, readily demonstrated in sera from HIV-1 infected individuals (31, 44) and animals immunized with recombinant envelope glycoproteins (1-3), has not always correlated with neutralizing activity (2, 31, 44). Results obtained with monoclonal antibodies have shown that while some of the monoclonal antibodies that block the binding of gp120 to CD4 possess neutralizing activity, others do not (4, 7, 16, 26, 33, 35, 43, 45). When the neutralizing activity of CD4 blocking monoclonal antibodies are compared to those directed to the principal neutralizing determinant (PND) located in the third variable domain (V3 domain) of gp120 (10, 39), the CD4 blocking antibodies appear to be significantly less potent. Thus, CD4 blocking monoclonal antibodies typically exhibit 50% inhibitory concentration values (IC_{50}) in the 1-10 μg/ml range (4, 16, 26, 33, 35, 43, 45) whereas PND directed monoclonal antibodies typically exhibit IC_{50} values in the 0.1 to 1.0 μg/ml range (23, 33, 42).

Subunit vaccines, based on gp120 or another viral protein, that can effectively induce antibodies that neutralize HIV are still being sought. However, to date no vaccine has not been effective in conferring protection against HIV infection.

DESCRIPTION OF THE BACKGROUND ART

Recombinant subunit vaccines are described in Berman *et al.*, PCT/US91/02250 (published as number WO91/15238 on 17 October 1991). See also, e.g. Hu *et al.*, *Nature* 328:721-724 (1987) (vaccinia virus-HIV envelope recombinant vaccine); Arthur *et al.*, *J. Virol.* 63(12): 5046-5053 (1989) (purified gp120); and Berman *et al.*, *Proc. Natl. Acad. Sci. USA* 85:5200-5204 (1988) (recombinant envelope glycoprotein gp120).

Numerous sequences for gp120 are known. The sequence of gp120 from the IIIB substrain of HIV-1_{LAI} referred to herein is that determined by Muesing *et al.*, "Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus, *Nature* 313:450-458 (1985). The sequences of gp120 from the NY-5, Jrcsf, Z6, Z321, and HXB2 strains of HIV-1 are listed by Myers *et al.*, "Human Retroviruses and AIDS; A compilation and analysis of nucleic acid and amino acid sequences," Los Alamos National Laboratory, Los Alamos, New Mexico (1992). The sequence of the Thai isolate A244 is provided by McCutchan *et al.*, "Genetic Variants of HIV-1 in Thailand," *AIDS Res. and Human Retroviruses* 8:1887-1895 (1992). The MN_{1984} clone is described by Gurgo *et al.*, "Envelope sequences of two new United States HIV-1 isolates," *Virol.* 164: 531-536 (1988). The amino acid sequence of this MN clone differs by approximately 2% from the MN-gp120 clone (MN_{GNE}) disclosed herein and obtained by Berman *et al.*

Each of the above-described references is incorporated herein by reference in its entirety.

SUMMARY OF THE INVENTION

The present invention provides a method for the rational design and preparation of vaccines based on HIV envelope polypeptides. This invention is based on the discovery that there are neutralizing epitopes in

the V2 and C4 domains of gp120, in addition to the neutralizing epitopes in the V3 domain. In addition, the amount of variation of the neutralizing epitopes is highly constrained, facilitating the design of an HIV subunit vaccine that can induce antibodies that neutralize a plurality of HIV strains for a given geographic region.

In one embodiment, the present invention provides a method for making an HIV gp120 subunit vaccine for a geographic region in which a neutralizing epitope in the V2 and/or C4 domains of gp120 of HIV isolates from the geographic region is determined and an HIV strain having gp120 which has a neutralizing epitope in the V2 or C4 domain which is common among isolates in the geographic region is selected and used to make the vaccine.

In a preferred embodiment of the method, neutralizing epitopes for the V2, V3, and C4 domains of gp120 from HIV isolates from the geographic region are determined. At least two HIV isolates having different neutralizing epitopes in the V2, V3, or C4 domain are selected and used to make the HIV gp120 subunit vaccine. Preferably, each of the selected isolates have one of the most common neutralizing epitopes for the V2, V3, or C4 domains.

The invention also provides a multivalent HIV gp120 subunit vaccine. The vaccine comprises gp120 from two isolates of HIV having at least one different neutralizing epitope. Preferably, the isolates have the most common neutralizing epitopes in the geographic region for one of the domains.

A DNA sequence of less than 5 kilobases encoding gp120 from preferred vaccine strains of HIV, GNE_8 and GNE_{16}, expression construct comprising the GNE_8-gp120 and GNE_{16}-gp120 encoding DNA under the transcriptional and translational control of a heterologous promoter, and

isolated GNE_8-gp120 and GNE_{16}-gp120 are also provided. The invention further provid s improved methods f r HIV serotyping in which epitopes in the V2 or C4 domains of gp120 are determined and provides immunogens (truncated gp120 sequences) which induce antibodies useful in the serotyping methods.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 describes inhibition of CD4 binding by monoclonal antibodies to recombinantly produced gp120 from the MN strain of HIV (MN-rgp120). Mice were immunized with MN-rgp120 and the resulting splenocytes were fused with the NP3X63.Ag8.653 cell line as described in Example 1. Thirty-five stable hybridoma clones, reactive with MN-rgp120 were identified by ELISA. Secondary screening revealed seven cell lines (1024, 1093, 1096, 1097, 1110, 1112, and 1027) secreting antibodies able to inhibit the binding of MN-rgp120 to biotin labeled recombinantly produced CD4 (rsCD4) in a ELISA using HRPO-strepavadin. Data obtained with monoclonal antibodies from the same fusion (1026, 1092, 1126) that failed to inhibit MN-rgp120 binding to CD4 is shown for purposes of comparison.

FIGURE 2 shows neutralizing activity of CD4-blocking monoclonal antibodies to MN-rgp120. Monoclonal antibodies that blocked the binding of MN-rgp120 to CD4 were screened for the capacity to inhibit the infection of MT2 cells by the MN strain of HIV-1 in vitro. Cell free virus was added to wells containing serially diluted antibodies and incubated at 4°C for 1 hr. After incubation, MT-2 cells were added to the wells and the cultures were then grown for 5 days at 37°C. Cell viability was then measured by addition of the colorimetric tetrazolium compound MTT as described in reference (35) of Example 1. The

optical densities f each well were measured at 540 nm using a microtit r plat reading spectrophotometer. Inhibition of virus infectivity was calculated by dividing the mean optical densities from wells containing monoclonal antibodies by the mean value of wells that received virus alone. Monoclonal antibodies that blocked CD4 binding are the same as those indicated in Figure Legend 1. Data from the V3-directed monoclonal antibody to MN-rgp120 (1034) is provided as a positive control. Data obtained with the V3 directed monoclonal antibody, 11G5, specific for the IIIB strain of HIV-1 (33) is shown as a negative control.

FIGURE 3 is a diagram of gp120 fragments used to localize the epitopes recognized by the CD4 blocking monoclonal antibodies to MN-rgp120. A series of fragments (A) corresponding to the V4 and C4 domains (B) (SEQ. ID. NO. 14) of the gene encoding MN-rgp120 were prepared by PCR. The gp120 gene fragments were fused to a fragment of the gene encoding Herpes Simplex Virus Type 1 glycoprotein D that encoded the signal sequence and 25 amino acids from the mature amino terminus. The chimeric genes were assembled into a mammalian cell expression vector (PRK5) that provided a CMV promoter, translational stop codons and an SV40 polyadenylation site. The embryonic human kidney adenocarcinoma cell line, 293s, was transfected with the resulting plasmid and recombinant proteins were recovered from growth conditioned cell culture medium. Fragments of MN-rgp120, expressed as HSV-1 Gd fusion proteins, were produced by transient transfection of 293s cells (Example 1). To verify expression, cells were metabolically labeled with [^{35}S]-methionine, and the resulting growth conditioned cell culture supernatants were immunoprecipitated (C) using a monoclonal antibody, 5B6, specific for the amino

terminus of HSV-1 Gd and fixed S. aureus. The immunoprecipitated proteins were resolved on 4 to 20 % acrylamide gradient gels using SDS-PAGE and visualized by autoradiography. The samples were: Lane 1, FMN.368-408; lane 2, FMN.368-451; lane 3, FMN.419-443; lane 4, FMN.414-451; lane 5, MN-rgp120. The gel demonstrated that the proteins were expressed and migrated at the expected molecular weights.

FIGURE 4 shows a C4 domain sequence comparison (SEQ. ID. Nos. 3-13). The C4 domain amino acid sequences of recombinant and virus derived gp120s used for monoclonal antibody binding studies were aligned starting the amino terminal cysteine. Amino acid positions are designated with respect to the sequence of MN-rgp120. Sequences of the LAI substrains, IIIB, BH10, Bru, HXB2, and HXB3 are shown for purposes of comparison.

FIGURE 5 shows sequences of C4 domain mutants of MN-rgp120 (SEQ. ID. Nos. 3 and 15-23). Nucleotide substitutions, resulting in the amino acid sequences indicated, were introduced into the C4 domain of MN-rgp120 gene using recombinant PCR. The resulting variants were assembled into the expression plasmid, pRK5, which was then transfected into 293s cells. The binding of monoclonal antibodies to the resulting C4 domain variants was then analyzed (Table 5) by ELISA.

FIGURE 6 illustrates the reactivity of monoclonal antibody 1024 with HIV-1_{LAI} substrains. The cell surface binding of the C4 domain reactive monoclonal antibody 1024 to H9 cells chronically infected with the IIIB, HXB2, HXB3, and HXB10 substrains of HIV-1 LAI or HIV-1MN was analyzed by flow cytometry. Cultures of virus infected cells were reacted with either monoclonal antibody 1024, a nonrelevant monoclonal antibody (control), or a broadly cross reactive monoclonal antibody (1026) raised against rgp120.

After washing away unbound monoclonal antibody, the cells were then labeled with fluorescein conjugated goat antibody t mouse IgG $(Fab')_2$, washed and fixed with paraformaldehyde. The resulting cells were analyzed for degree of fluorescence intensity using a FACSCAN (Becton Dickenson, Fullerton, CA). Fluorescence was measured as mean intensity of the cells expressed as mean channel number plotted on a log scale.

FIGURE 7 shows the determination of the binding affinity of monoclonal antibodies for MN-rgp120. CD4 blocking monoclonal antibodies raised against MN-rgp120 (1024 and 1097) or IIIB-rgp120 (13H8 and 5C2) were labeled with [^{125}I] and binding titrations using MN-rgp120 (A and B) or IIIB-rgp120 (C and D) were carried out as described in the Example 1. A, binding of monoclonal antibody 1024; B binding of monoclonal antibody 1097; C, binding of monoclonal antibody 13H8; and D binding of monoclonal antibody 5C2.

FIGURE 8 shows the correlation between gp120 binding affinity (K_d) and neutralizing activity (IC50) of monoclonal antibodies to the C4 domain of MN-rgp120. Binding affinities of monoclonal antibodies to the C4 domain of gp120 were determined by Scatchard analysis (Figure 9, Table 5). The resulting values were plotted as a function of the log of their neutralizing activities (IC_{50}) determined in Figure 2 and Table 6.

FIGURE 9 depicts the amino acid sequence of the mature envelope glycoprotein (gp120) from the MN_{GNE} clone of the MN strain of HIV-1 (SEQ. ID. NO. 1). Hypervariable domains are from 1-29 (signal sequence), 131-156, 166-200,305-332, 399-413, and 460-469. The V and C regions are indicated (according to Modrow *et al.*, *J. Virology* 61(2):570 (1987). Potential glycosylation sites are marked with a (*).

FIGURE 10 depicts the amino acid sequence of a fusi n protein of the residues 41-511 of the mature envelope glycoprotein (gp120) from the MN_{GNE} clone of the MN strain of HIV-1, and the gD-1 amino terminus from the herpes simplex glycoprotein gD-1. (SEQ. ID. NO. 2). The V and C regions are indicated (according to Modrow *et al.*, *J. Virology* 61(2):570 (1987). Potential glycosylation sites are marked with a (*).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the rational design and preparation of vaccines based on HIV envelope polypeptides. This invention is based on the discovery that there are neutralizing epitopes in the V2 and C4 domains of gp120, in addition to the neutralizing epitopes in the V3 domain. Although the amino acid sequences of the neutralizing epitopes in the V2, V3, and C4 domains are variable, it has now been found that the amount of variation is highly constrained. The limited amount of variation facilitates the design of an HIV subunit vaccine that can induce antibodies that neutralize the most common HIV strains for a given geographic region. In particular, the amino acid sequence of neutralizing epitopes in the V2, V3, and C4 domains for isolates of a selected geographic region is determined. gp120 from isolates having the most common neutralizing epitope sequences are utilized in the vaccine.

The invention also provides a multivalent gp120 subunit vaccine wherein gp120 present in the vaccine is from at least two HIV isolates which have different amino acid sequences for a neutralizing epitope in the V2, V3, or C4 domain of gp120. The invention further provides improved methods for HIV serotyping in which epitopes in the V2 or C4 domains of gp120 are

determined and provides immunogens which induce antibodies useful in the serotyping methods.

The term "subunit vaccine" is used herein, as in the art, to refer to a viral vaccine that does not contain virus, but rather contains one or more viral proteins or fragments of viral proteins. As used herein, the term "multivalent" means that the vaccine contains gp120 from at least two HIV isolates having different amino acid sequences for a neutralizing epitope.

Vaccine Design Method

The vaccine design method of this invention is based on the discovery that there are neutralizing epitopes in the V2 and C4 domains of gp120, in addition to those found in the principal neutralizing domain (PND) in the V3 domain. Selecting an HIV isolate with appropriate neutralizing epitopes in the V2 and/or C4 domains provides a vaccine that is designed to induce immunity to the HIV isolates present in a selected geographic region. In addition, although the amino acid sequence of the V2, V3, and C4 domains containing the neutralizing epitopes is variable, the amount of variation is highly constrained, facilitating the design of a multivalent vaccine which can neutralize a plurality of the most common HIV strains for a given geographic region.

The method for making an HIV gp120 subunit vaccine depends on the use of appropriate strains of HIV for a selected geographic region. Appropriate strains of HIV for the region are selected by determining the neutralizing epitopes for HIV isolates and the percentage of HIV infections attributable to each strain present in the region. HIV strains which have the most common neutralizing epitopes in the V2 or C4 domains in the geographic region are selected.

Preferably, isolates that confer protection against the most common neutralizing epitopes in the V2, V3, and C4 domains for a geographic region are selected.

One embodiment of the method for making an HIV gp120 subunit vaccine from appropriate strains of HIV for a geographic region comprises the following steps. A neutralizing epitope in the V2 or C4 domain of gp120 of HIV isolates from the geographic region is determined. An HIV strain having gp120 with a neutralizing epitope in the V2 or C4 domain that is common among HIV isolates in the geographic region is selected. gp120 from the selected isolate is used to make an HIV gp120 subunit vaccine.

In another embodiment of the method, the neutralizing epitopes in the V2, V3, and C4 domains of gp120 from HIV isolates from the geographic region are determined. At least two HIV isolates having different neutralizing epitopes in the V2, V3, or C4 domain are selected and used to make an HIV gp120 subunit vaccine. Preferably, the vaccine contains gp120 from at least the two or three HIV strains having the most common neutralizing epitopes for the V2, V3, or C4 domains. More preferably, the vaccine contains gp120 from sufficient strains so that at least about 50%, preferably about 70%, more preferably about 80% or more of the neutralizing epitopes for the V2, V3, and C4 domains in the geographic region are included in the vaccine. The location of the neutralizing epitopes in the V3 region are well known. The location of the neutralizing epitopes in the V2 and C4 regions are described hereinafter.

Each of the steps of the method are described in detail below.

Determining neutralizing epitopes

The first step in designing a vaccine for a selected geographic regi n is to determine the neutralizing epitopes in the gp120 V2 and/or C4 domains. In a preferred embodiment, neutralizing epitopes in the V3 domain (the principal neutralizing domain) are also determined. The location of neutralizing epitopes in the V3 domain is well known. Neutralizing epitopes in the V2 and C4 domains have now been found to be located between about residues 163 and 200 and between about residues 420 and 440, respectively. In addition, the critical residues for antibody binding are residues 171, 173, 174, 177, 181, 183, 187, and 188 in the V2 domain and residues 429 and 432 in the C4 domain, as described in detail in the Examples.

The neutralizing epitopes for any isolate can be determined by sequencing the region of gp120 containing the neutralizing epitope. Alternatively, when antibodies specific for the neutralizing epitope, preferably monoclonal antibodies, are available the neutralizing epitope can be determined by serological methods as described hereinafter. A method for identification of additional neutralizing epitopes in gp120 is described hereinafter.

When discussing the amino acid sequences of various isolates and strains of HIV, the most common numbering system refers to the location of amino acids within the gp120 protein using the initiator methionine residue as position 1. The amino acid numbering reflects the mature HIV-1 gp120 amino acid sequence as shown by Figures 9 and Fig. 10 [SEQ. ID Nos. 1 and 2]. For gp120 sequences derived from other HIV isolates and which include their native HIV N-terminal signal sequence, numbering may differ. Although the nucleotide and amino acid residue numbers may not be applicable in other strains where upstream deletions or

insertions change the length of the viral genome and gp120, the regi n encoding the portions of gp120 is readily identified by reference to the teachings herein. The variable (V) domains and conserved (C) domains of gp120 are specified according to the nomenclature of Modrow *et al.* "Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: predictions of antigenic epitopes in conserved and variable regions," *J. Virol.* **61**:570-578 (1987).

The first step in identifying the neutralizing epitopes for any region of gp120 is to immunize an animal with gp120 to induce anti-gp120 antibodies. The antibodies can be polyclonal or, preferably, monoclonal. Polyclonal antibodies can be induced by administering to the host animal an immunogenic composition comprising gp120. Preparation of immunogenic compositions of a protein may vary depending on the host animal and the protein and is well known. For example, gp120 or an antigenic portion thereof can be conjugated to an immunogenic substance such as KLH or BSA or provided in an adjuvant or the like. The induced antibodies can be tested to determine whether the composition is specific for gp120. If a polyclonal antibody composition does not provide the desired specificity, the antibodies can be fractionated by ion exchange chromatography and immunoaffinity methods using intact gp120 or various fragments of gp120 to enhance specificity by a variety of conventional methods. For example, the composition can be fractionated to reduce binding to other substances by contacting the composition with gp120 affixed to a solid substrate. Those antibodies which bind to the substrate are retained. Fractionation techniques using antigens affixed to a variety of solid substrates such as affinity chromatography materials

including Sephadex, Sepharose and the like are well known.

Monoclonal anti-gp120 antibodies can be produced by a number of conventional methods. A mouse can be injected with an immunogenic composition containing gp120 and spleen cells obtained. Those spleen cells can be fused with a fusion partner to prepare hybridomas. Antibodies secreted by the hybridomas can be screened to select a hybridoma wherein the antibodies neutralize HIV infectivity, as described hereinafter. Hybridomas that produce antibodies of the desired specificity are cultured by standard techniques.

Infected human lymphocytes can be used to prepare human hybridomas by a number of techniques such as fusion with a murine fusion partner or transformation with EBV. In addition, combinatorial libraries of human or mouse spleen can be expressed in E. coli to produce the antibodies. Kits for preparing combinatorial libraries are commercially available. Hybridoma preparation techniques and culture methods are well known and constitute no part of the present invention. Exemplary preparations of monoclonal antibodies are described in the Examples.

Following preparation of anti-gp120 monoclonal antibodies, the antibodies are screened to determine those antibodies which are neutralizing antibodies. Assays to determine whether a monoclonal antibody neutralizes HIV infectivity are well known and are described in the literature. Briefly, dilutions of antibody and HIV stock are combined and incubated for a time sufficient for antibody binding to the virus. Thereafter, cells that are susceptible to HIV infection are combined with the virus/antibody mixture and cultured. MT-2 cells or H9 cells are susceptible to infection by most HIV strains that are adapted for

growth in the laboratory. Activated peripheral blood mononuclear cells (PBMCs) or macrophages can be infected with primary isolates (isolates from a patient specimens which have not been cultured in T-cell lines or transformed cell lines). Daar et al, *Proc. Natl. Acad. Sci. USA* 87:6574-6578 (1990) describe methods for infecting cells with primary isolates.

After culturing the cells for about five days, the number of viable cells is determined, as by measuring metabolic conversion of the formazan MTT dye. The percentage of inhibition of infectivity is calculated to determine those antibodies that neutralize HIV. An exemplary preferred procedure for determining HIV neutralization is described in the Examples.

Those monoclonal antibodies which neutralize HIV are used to map the epitopes to which the antibodies bind. To determine the location of a gp120 neutralizing epitope, neutralizing antibodies are combined with fragments of gp120 to determine the fragments to which the antibodies bind. The gp120 fragments used to localize the neutralizing epitopes are preferably made by recombinant DNA methods as described hereinafter and exemplified in the Examples. By using a plurality of fragments, each encompassing different, overlapping portions of gp120, an amino acid sequence encompassing a neutralizing epitope to which a neutralizing antibody binds can be determined. A preferred exemplary determination of the neutralizing epitopes to which a series of neutralizing antibodies binds is described in detail in the Examples.

This use of overlapping fragments can narrow the location of the epitope to a region of about 20 to 40 residues. To confirm the location of the epitope and narrow the location to a region of about 5 to 10 residues, site-directed mutagenicity studies are preferably performed. Such studies can also determine

the critical residues for binding of neutralizing antibodies. A preferred exemplary site-directed mutagenicity procedure is described in the Examples.

To perform site-directed mutagenicity studies, recombinant PCR techniques can be utilized to introduce single amino acid substitutions at selected sites into gp120 fragments containing the neutralizing epitope. Briefly, overlapping portions of the region containing the epitope are amplified using primers that incorporate the desired nucleotide changes. The resultant PCR products are annealed and amplified to generate the final product. The final product is then expressed to produce a mutagenized gp120 fragment. Expression of DNA encoding gp120 or a portion thereof is described hereinafter and exemplified in the Examples.

In a preferred embodiment described in Example 1, the gp120 fragments are expressed in mammalian cells that are capable of expression of gp120 fragments having the same glycolsylation and disulfide bonds as native gp120. The presence of proper glycolsylation and disulfide bonds provides fragments that are more likely to preserve the neutralizing epitopes than fragments that are expressed in E. coli, for example, which lack disulfide bonds and glycosylation or are chemically synthesized which lack glycolsylation and may lack disulfide bonds.

Those mutagenized gp120 fragments are then used in an immunoassay using gp120 as a control to determine the mutations that impair or eliminate binding of the neutralizing antibodies. Those critical amino acid residues form part of the neutralizing epitope that can only be altered in limited ways without eliminating the epitope. Each alteration that preserves the epitope can be determined. Such mutagenicity studies demonstrate the variations in the amino acid sequence

of the neutralizing epitope that provide equivalent or diminished binding by neutralizing antibodies or eliminate antibody binding. Although the amino acid sequence of gp120 used in the vaccine preferably is identical to that of a selected HIV isolate for the given geographic region, alterations in the amino acid sequence of neutralizing epitope that are suitable for use in a vaccine can be determined by such studies.

Once a neutralizing epitope is localized to a region of ten to twenty amino acids of gp120, the amino acid sequence of corresponding neutralizing epitopes of other HIV isolates can be determined by identifying the corresponding portion of the gp120 amino acid sequence of the isolate.

Once the neutralizing epitopes for a given region of gp120 are determined, the amino acid sequence of HIV isolates for the geographic region are determined. The complete amino acid sequence for numerous isolates has been determined and is available from numerous journal articles and in databases. In such cases, determination of the amino acid sequence of HIV isolates for the geographic region involves looking up the sequence in an appropriate database or journal article. However, for some isolates, the amino acid sequence information does not include the sequence of the V2 or C4 domains.

When the amino acid sequence of a region of interest for a given isolate is not known, the amino acid sequence can be determined by well known methods. Methods for determining the amino acid sequence of a protein or peptide of interest are well known and are described in numerous references including Maniatis et al., Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory (1984). In addition, automated instruments which sequence proteins are commercially available.

Alternatively, the nucleotide sequence of DNA encoding gp120 or a relevant portion of gp120 can be determined and the amino acid sequence of gp120 can be deduced. Methods for amplifying gp120-encoding DNA from HIV isolates to provide sufficient DNA for sequencing are well known. In particular, Ou et al, *Science* **256**:1165-1171 (1992); Zhang et al. *AIDS* **5**:675-681 (1991); and Wolinsky *Science* **255**:1134-1137 (1992) describe methods for amplifying gp120 DNA. Sequencing of the amplified DNA is well known and is described in Maniatis et al., Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory (1984), and Horvath et al., An Automated DNA Synthesizer Employing Deoxynucleoside 3'-Phosphoramidites, Methods in Enzymology 154: 313-326, (1987), for example. In addition, automated instruments that sequence DNA are commercially available.

In a preferred embodiment, the isolate is a patient isolate which has not been passaged in culture. It is known that following passage in T-cells, HIV isolates mutate and isolates best suited for growth under cell culture conditions are selected. For example, cell culture strains of HIV develop the ability to form syncytia. Therefore, preferably the amino acid sequence of gp120 is determined from a patient isolate prior to growth in culture. Generally, DNA from the isolate is amplified to provide sufficient DNA for sequencing. The deduced amino acid sequence is used as the amino acid sequence of the isolate, as described hereinbefore.

To determine the percentage each isolate constitutes of total HIV that infects individuals in the geographic region, standard epidemiological methods are used. In particular, sufficient isolates are

sequenced to ensure confidence that the percentage of each isolate in the g ographic region has been determined. For example, Ichimura et al, *AIDS Res. Hum. Retroviruses* 10:263-269 (1994) describe an epidemiological study in Thailand that determined that there are two strains of HIV present in the region. HIV strains have only recently been present in Thailand and Thailand, therefore has the most homogenous population of HIV isolates known to date. The study sequenced 23 isolates from various parts of the country and determined that only two different amino acid sequences were present in the isolates.

In contrast, HIV has been infecting individuals in Africa for the longest period of any geographic region. In Africa, each of the most common isolates probably constitutes about 5% of the population. In such cases, more isolates would need to be sequenced to determine the percentage each isolate constitutes of the population. Population studies for determining the percentage of various strains of HIV, or other viruses, present in a geographic region are well known and are described in, for example, Ou et al, *Lancet* 341:1171-1174 (1993); Ou et al, *AIDS Res. Hum. Retroviruses* 8:1471-1472 (1992); and McCutchan et al., *AIDS Res. Hum. Retroviruses* 8:1887-1895 (1992).

In the United States and western Europe, probably about two to four different neutralizing epitopes in each of the V2, V3, and C4 domains constitute 50 to 70% of the neutralizing epitopes for each domain in the geographic region, as described more fully hereinafter.

Selection method

Once the amino acid sequence of neutralizing epitopes for strains in a region are determined, gp120 from an HIV strain having gp120 that has an amino acid sequence for a neutralizing epitope in the V2 or C4

domain which sequence is one of the most common in the g ographic region is selected. One of the most common neutralizing epitope amino acid sequences means that the strain has an amino acid sequence for at least one neutralizing epitope that is occurs among the most frequently for HIV isolates in the geographic region and thus is present as a significant percentage of the population. For example, if there are three sequences for a neutralizing epitope that constitute 20, 30, and 40 percent of the sequences for that epitope in the region and the remainder of the population is comprised by 2 to 4 other sequences, the three sequences are the most common. Therefore, in African countries, if each of several amino acid sequences constitute about 5% of the sequences for a neutralizing epitope and the remainder of the sequences each constitute less than 1% of the population, the isolates that constitute 5% of the population are the most common.

Preferably, isolates having the most common amino acid sequences for a neutralizing epitope are chosen. By the most common is meant that the sequences occur most frequently in the geographic region. For example, in the United States, the MN isolate has a C4 neutralizing epitope that comprises at least about 45% of the population. The GNE_8 isolate has a C4 neutralizing epitope that comprises at least about 45% of the population. Thus either isolate has the most common C4 neutralizing epitope in the region. When gp120 from each isolate is combined in a vaccine, greater than about 90% of the C4 neutralizing epitope sequences are present in the vaccine. In addition, the amino acid sequences for the V3 neutralizing epitope in the MN and GNE_8 isolates are substantially similar and comprise about 60% of the population. Therefore, those strains have the two most common neutralizing epitopes for the V3 domain. In the V2 region, the MN isolate

amino acid sequences comprises about 10% of the population, and the GNE_8 isolate amino acid sequences comprises about 60% of the population. Therefore, the GNE_8 strain has the most common neutralizing epitope for the region and the two strains together comprise the two most common neutralizing epitopes for the region. A multivalent gp120 subunit vaccine containing the two isolates contains amino acid sequences for epitopes that constitute about 70% of the V2 domain, about 60% of the V3 domain, and about 90% of the C4 domain for the United States.

In a preferred embodiment of the method, one or more HIV isolates having an amino acid sequence for a neutralizing epitope in the V2 and/or C4 domains that constitute at least about 50% of the population for a selected geographic region are selected. In a more preferred embodiment, isolates having the most common neutralizing epitopes in the V3 domain are also included in the vaccine.

As is clear, once the most common amino acid sequences for the neutralizing epitopes in the V2, V3, and C4 domains are known, an isolate having a common epitope for each region is preferably selected. That is, when only two or three isolates are used for the vaccine, it is preferable to select the isolate for common epitopes in each region, rather than selecting an isolate by analysis of a single region.

In a more preferred embodiment, gp120 from isolates having epitopes that constitute at least 50% of the population for the geographic region for V2, V3, and C4 domains are present in the vaccine. More preferably, the isolates have epitopes that constitute at least 60% of the population for the geographic region for the three domains. Most preferably, 70% or more are included.

In another preferred embodiment, the entire amino acid sequence of the V2 and C4 domains is determined in the selection process. In addition to selecting common sequences for the neutralizing epitopes, isolates having unusual polymorphisms elsewhere in the region are preferably not used for the vaccine isolates.

Vaccine preparation

gp120 from the selected HIV isolate(s) is used to make a subunit vaccine, preferably a multivalent subunit vaccine. Preparation of gp120 for use in a vaccine is well known and is described hereinafter. With the exception of the use of the selected HIV isolate, the gp120 subunit vaccine prepared in the method does not differ from gp120 subunit vaccines of the prior art.

As with prior art gp120 subunit vaccines, gp120 at the desired degree of purity and at a sufficient concentration to induce antibody formation is mixed with a physiologically acceptable carrier. A physiologically acceptable carrier is nontoxic to a recipient at the dosage and concentration employed in the vaccine. Generally, the vaccine is formulated for injection, usually intramuscular or subcutaneous injection. Suitable carriers for injection include sterile water, but preferably are physiologic salt solutions, such as normal saline or buffered salt solutions such as phosphate buffered saline or ringer's lactate. The vaccine generally contains an adjuvant. Useful adjuvants include QS21 which stimulates cytotoxic T-cells and alum (aluminum hydroxide adjuvant). Formulations with different adjuvants which enhance cellular or local immunity can also be used.

Addition excipients that can be present in the vaccine include low molecular weight polypeptides (less than about 10 residues), proteins, amino acids,

carbohydrates including glucose or dextrans, chelating agents such as EDTA, and other excipients.

The vaccine can also contain other HIV proteins. In particular, gp41 or the extracellular portion of gp41 can be present in the vaccine. Since gp41 has a conserved amino acid sequence, the gp41 present in the vaccine can be from any HIV isolate. gp160 from an isolate used in the vaccine can replace gp120 in the vaccine or be used together with gp120 from the isolate. Alternatively, gp160 from an isolate having a different neutralizing epitope than those in the vaccine isolates can additionally be present in the vaccine.

Vaccine formulations generally include a total of about 300 to 600 μg of gp120, conveniently in about 1.0 ml of carrier. The amount of gp120 for any isolate present in the vaccine will vary depending on the immunogenicity of the gp120. For example, gp120 from the Thai strains of HIV are much less immunogenic than gp120 from the MN strain. If the two strains were to be used in combination, empirical titration of the amount of each virus would be performed to determine the percent of the gp120 of each strain in the vaccine. For isolates having similar immunogenicity, approximately equal amounts of each isolate's gp120 would be present in the vaccine. For example, in a preferred embodiment, the vaccine includes gp120 from the MN, GNE_8, and GNE_{16} strains at concentrations of about 300 μg per strain in about 1.0 ml of carrier. Methods of determining the relative amount of an immunogenic protein in multivalent vaccines are well known and have been used, for example, to determine relative proportions of various isolates in multivalent polio vaccines.

The vaccines of this invention are administered in the same manner as prior art HIV gp120 subunit

vaccines. In particular, the vaccines are generally administered at 0, 1, and at 6, 8 or 12 months, depending on the protocol. Following the immunization procedure, annual or bi-annual boosts can be administered. However, during the immunization process and thereafter, neutralizing antibody levels can be assayed and the protocol adjusted accordingly.

The vaccine is administered to uninfected individuals. In addition, the vaccine can be administered to seropositive individuals to augment immune response to the virus, as with prior art HIV vaccines. It is also contemplated that DNA encoding the strains of gp120 for the vaccine can be administered in a suitable vehicle for expression in the host. In this way, gp120 can be produced in the infected host, eliminating the need for repeated immunizations. Preparation of gp120 expression vehicles is described hereinafter.

Production of gp120

gp120 in the vaccine can be produced by any suitable means, as with prior art HIV gp120 subunit vaccines. Recombinantly-produced or chemically synthesized gp120 is preferable to gp120 isolated directly from HIV for safety reasons. Methods for recombinant production of gp120 are described below.

DNA Encoding GNE_8 and GNE_{16} gp120 and the resultant proteins

The present invention also provides novel DNA sequences encoding gp120 from the GNE_8 and GNE_{16} isolates which can be used to express gp120 and the resultant gp120 proteins. A nucleotide sequence of less than about 5 kilobases (Kb), preferably less than about 3 Kb having the nucleotide sequence illustrated in Tables 1 and 2, respectively, encodes gp120 from the GNE_8 and

GNE_{16} isolates. The sequences of the genes and the encoded proteins are shown below in Tables 1-3. In particular, Table 1 illustrates the nucleotide sequence (SEQ. ID. NO. 27) and the predicted amino acid sequence (SEQ. ID. NO. 28) of the GNE_8 isolate of HIV. The upper sequence is the coding strand. The table also illustrates the location of each of the restriction sites.

TABLE 1

```
                                                    hgiCI
                                                    banI                                                scfI
                                                    bsp1286                                             pstI
                                                    bmyI          styI                  scfI          bsgI
  1 ATGATAGTGA AGGGGATCAG GAAGAATTGT CAGCACTTGT GGAGATGGGG CACCATGCTC CTTGGGATGT TGATGATCTG TAGTGCTGCA GAAAAATTGT
    TACTATCACT TCCCCTAGTC CTTCTTAACA GTCGTGAACA CCTCTACCCC GTGGTACGAG GAACCCTACA ACTACTAGAC ATCACGACGT CTTTTTAACA
  1 M  I  V  K   G  I  R   K  N  C   Q  H  L  W   R  W  G   T  M  L   L  G  M  L   M  I  C   S  A  A   E  K  L  W

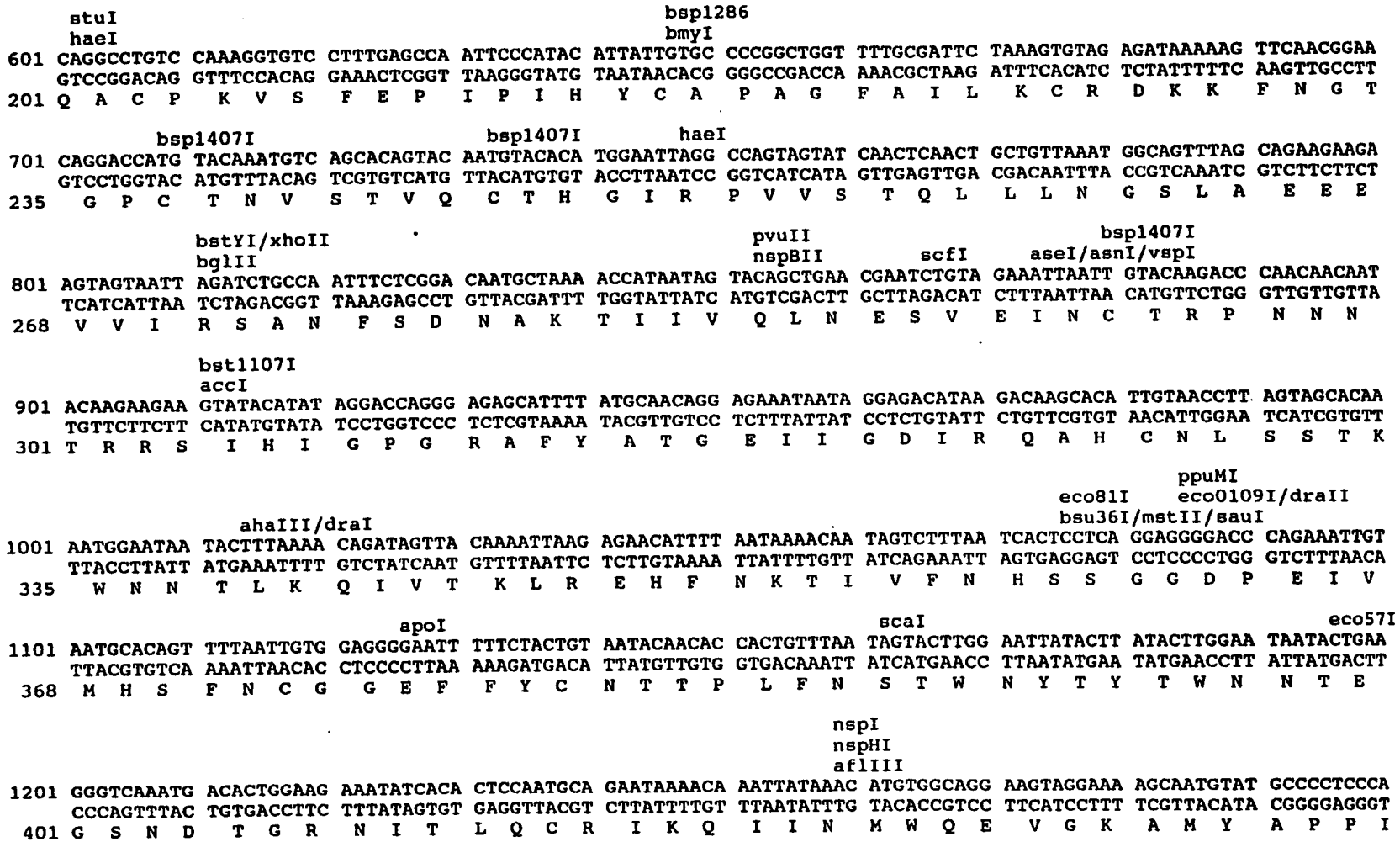
                        kpnI
                        hgiCI
                        banI
                        asp718
                        acc65I                                                          ndeI
101 GGGTCACAGT CTATTATGGG GTACCTGTGT GGAAAGAAGC AACCACCACT CTATTTTGTG CATCAGATGC TAAAGCATAT GATACAGAGG TACATAATGT
    CCCAGTGTCA GATAATACCC CATGGACACA CCTTTCTTCG TTGGTGGTGA GATAAAACAC GTAGTCTACG ATTTCGTATA CTATGTCTCC ATGTATTACA
 35   V  T  V   Y  Y  G   V  P  V  W   K  E  A   T  T  T   L  F  C  A   S  D  A   K  A  Y   D  T  E  V   H  N  V

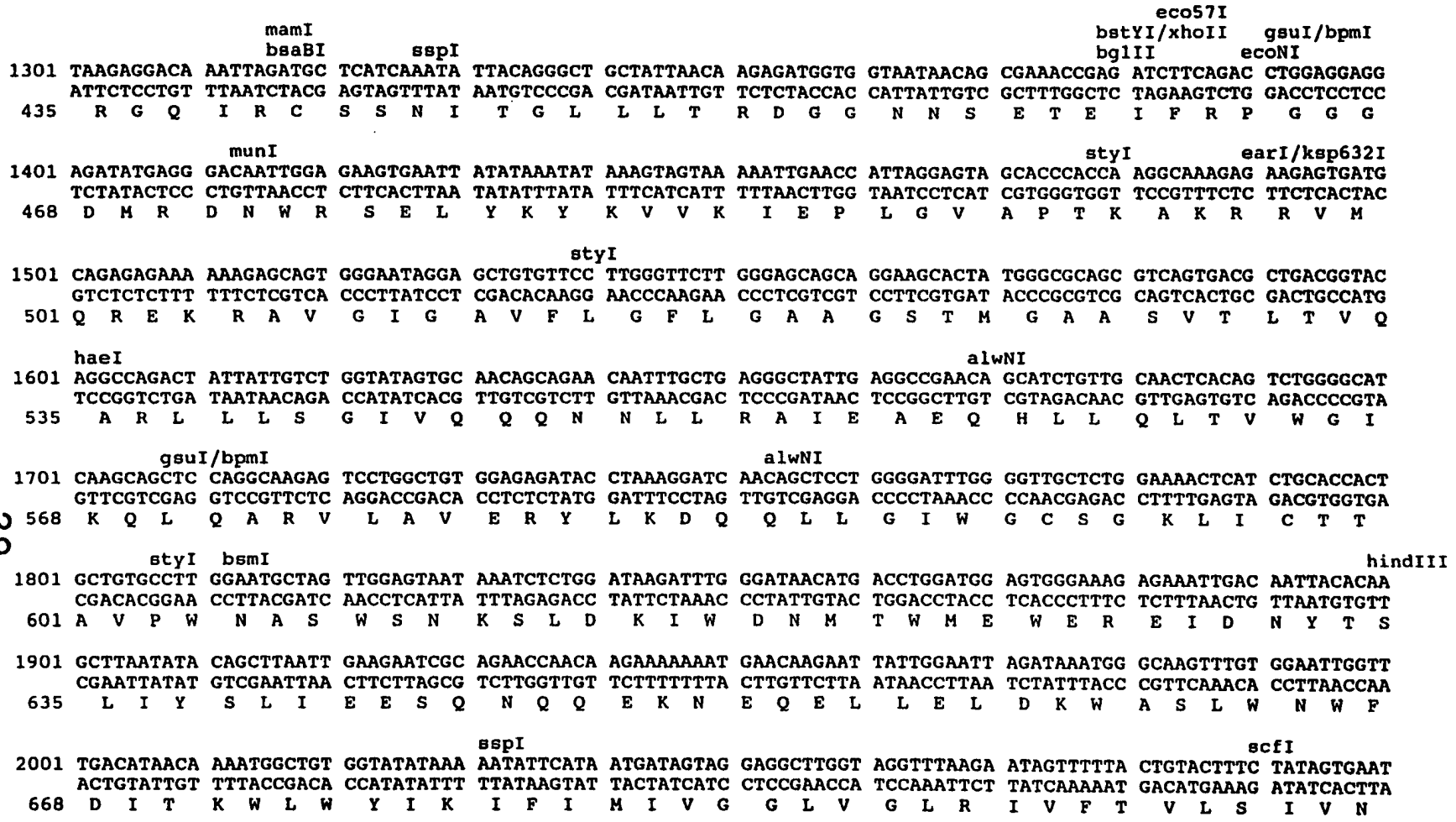
                                                                                                nspI
                                                                                                nspHI
             nspI                                                                               aflIII
             nspHI                                                                 apoI
201 TTGGGCCACA CATGCCTGTG TACCCACAGA CCCCAACCCA CAAGAAATAG GATTGGAAAA TGTAACAGAA AATTTTAACA TGTGGAAAAA TAACATGGTA
    AACCCGGTGT GTACGGACAC ATGGGTGTCT GGGGTTGGGT GTTCTTTATC CTAACCTTTT ACATTGTCTT TTAAAATTGT ACACCTTTTT ATTGTACCAT
 68  W  A  T   H  A  C  V   P  T  D   P  N  P   Q  E  I  G   L  E  N   V  T  E   N  F  N  M   W  K  N   N  M  V

          ppu10I
          nsiI/avaIII                              hindIII                                 draIII          ahaIII/draI
301 GAACAGATGC ATGAGGATAT AATCAGTTTA TGGGATCAAA GCTTAAAGCC ATGTGTAAAA TTAACCCCAC TATGTGTTAC TTTAAATTGC ACTGATTTGA
    CTTGTCTACG TACTCCTATA TTAGTCAAAT ACCCTAGTTT CGAATTTCGG TACACATTTT AATTGGGGTG ATACACAATG AAATTTAACG TGACTAAACT
101 E  Q  M  H   E  D  I   I  S  L   W  D  Q  S   L  K  P   C  V  K   L  T  P  L   C  V  T   L  N  C   T  D  L  K

                          pvuII
                   speI   nspBII
401 AAAATGCTAC TAATACCACT AGTAGCAGCT GGGGAAAGAT GGAGAGAGGA GAAATAAAAA ACTGCTCTTT CAATGTCACC ACAAGTATAA GAGATAAGAT
    TTTTACGATG ATTATGGTGA TCATCGTCGA CCCCTTTCTA CCTCTCTCCT CTTTATTTTT TGACGAGAAA GTTACAGTGG TGTTCATATT CTCTATTCTA
135   N  A  T   N  T  T   S  S  S  W   G  K  M   E  R  G   E  I  K  N   C  S  F   N  V  T   T  S  I  R   D  K  M

                                                                  scfI
501 GAAGAATGAA TATGCACTTT TTTATAAACT TGATGTAGTA CCAATAGATA ATGATAATAC TAGCTATAGG TTGATAAGTT GTAACACCTC AGTCATTACA
    CTTCTTACTT ATACGTGAAA AAATATTTGA ACTACATCAT GGTTATCTAT TACTATTATG ATCGATATCC AACTATTCAA CATTGTGGAG TCAGTAATGT
168  K  N  E   Y  A  L  F   Y  K  L   D  V  V   P  I  D  N   D  N  T   S  Y  R   L  I  S  C   N  T  S   V  I  T
```





```
                                                                  avaI
2101 AGAGTTAGGA AGGGATACTC ACCATTATCG TTCCAGACCC ACCTCCCAGC CCCGAGGGGA CTCGACAGGC CCGAAGGAAC CGAAGAAGAA GGTGGAGAGC
     TCTCAATCCT TCCCTATGAG TGGTAATAGC AAGGTCTGGG TGGAGGGTCG GGGCTCCCCT GAGCTGTCCG GGCTTCCTTG GCTTCTTCTT CCACCTCTCG
701  R  V  R  K   G  Y  S   P  L  S   F  Q  T  H   L  P  A   P  R  G   L  D  R  P   E  G  T   E  E  E   G  G  E  R

                                                                     bspMI
                                                                  salI
                                                                  hincII/hindII                eco57I
            xcmI                                                                               earI/ksp632I
          bstYI/xhoII                               munI          accI
2201 GAGACAGAGA CAGATCCAGT CGATTAGTGG ATGGATTCTT AGCAATTGTC TGGGTCGACC TGCGGAGCCT GTGCCTCTTC AGCTACCACC GCTTGAGAGA
     CTCTGTCTCT GTCTAGGTCA GCTAATCACC TACCTAAGAA TCGTTAACAG ACCCAGCTGG ACGCCTCGGA CACGGAGAAG TCGATGGTGG CGAACTCTCT
735   D  R  D   R  S  S   R  L  V  D   G  F  L   A  I  V   W  V  D  L   R  S  L   C  L  F   S  Y  H  R   L  R  D

                                                                             sspI              scfI
2301 CTTACTCTTG ATTGCAGCGA GGATTGTGGA ACTTCTGGGA CGCAGGGGGT GGGAAGCCCT CAAATATTGG TGGAATCTCC TACAGTATTG GATTCAGGAA
     GAATGAGAAC TAACGTCGCT CCTAACACCT TGAAGACCCT GCGTCCCCCA CCCTTCGGGA GTTTATAACC ACCTTAGAGG ATGTCATAAC CTAAGTCCTT
768  L  L  L   I  A  A  R   I  V  E   L  L  G   R  R  G  W   E  A  L   K  Y  W   W  N  L  L   Q  Y  W   I  Q  E

                                                       alwNI
2401 CTAAAGAATA GTGCTGTTAG CTTGCTCAAT GCCACAGCCA TAGCAGTAGC TGAGGGAACA GATAGGGTTA TAGAAATAGT ACAAAGAGCT TATAGAGCTA
     GATTTCTTAT CACGACAATC GAACGAGTTA CGGTGTCGGT ATCGTCATCG ACTCCCTTGT CTATCCCAAT ATCTTTATCA TGTTTCTCGA ATATCTCGAT
801  L  K  N  S   A  V  S   L  L  N   A  T  A  I   A  V  A   E  G  T   D  R  V  I   E  I  V   Q  R  A   Y  R  A  I

2501 TTCTCCACAT ACCCACACGA ATAAGACAGG GCTTGGAAAG GGCTTTGCTA TAA
     AAGAGGTGTA TGGGTGTGCT TATTCTGTCC CGAACCTTTC CCGAAACGAT ATT
835   L  H  I   P  T  R   I  R  Q  G   L  E  R   A  L  L   O
```

Table 2 illustrates the nucleotide sequence and the predicted amino acid sequence of the GNE_{16} isolate of HIV. The upper sequence is the coding strand. The table also illustrates the location of each of the restriction sites. The first four pages of the table are from one clone of the gene and the second three pages of the table are from another clone of the gene. The sequences of the clones differ by about 2%. (The nucleotide sequences are SEQ. ID. NOs. 28 and 29, respectively. The amino acid sequences are SEQ. ID. NOs. 30 and 31, respectively.) It is noted that each of the sequences includes a stop codon. A gene sequence that encodes full length gp120 can be made by repairing one of the sequences.

TABLE 2

```
                                                        hgiCI
                                                        banI                                               scfI
                                                        bsp1286                                            pstI
                                                        bmyI        styI                      scfI         bsgI
  1 ATGAGAGTGA AGGGGATCAG GAGGAATTAT CAGCACTTGT GGAGATGGGG CACCATGCTC CTTGGGATAT TGATGATCTG TAGTGCTGCA GGGAAATTGT
    TACTCTCACT TCCCCTAGTC CTCCTTAATA GTCGTGAACA CCTCTACCCC GTGGTACGAG GAACCCTATA ACTACTAGAC ATCACGACGT CCCTTTAACA
  1 M  R  V  K   G  I  R   R  N  Y   Q  H  L  W   R  W  G   T  M  L   L  G  I  L   M  I  C   S  A  A   G  K  L  W

                       kpnI
                       hgiCI
                       banI
                       asp718
                       acc65I                                                     ndeI
101 GGGTCACAGT CTATTATGGG GTACCTGTGT GGAAAGAAAC AACCACCACT CTATTTTGTG CATCAGATGC TAAAGCATAT GATACAGAGA TACATAATGT
    CCCAGTGTCA GATAATACCC CATGGACACA CCTTTCTTTG TTGGTGGTGA GATAAAACAC GTAGTCTACG ATTTCGTATA CTATGTCTCT ATGTATTACA
 35  V  T  V   Y  Y  G   V  P  V  W   K  E  T   T  T  T   L  F  C  A   S  D  A   K  A  Y   D  T  E  I   H  N  V

                                                                                        nspI
            nspI                                                                        nspHI
            nspHI                                                           apoI        aflIII
201 TTGGGCCACA CATGCCTGTG TACCCACAGA CCCCAACCCA CAAGAAGTAG TATTGGAAAA TGTGACAGAA AATTTTAACA TGTGGAAAAA TAACATGGTG
    AACCCGGTGT GTACGGACAC ATGGGTGTCT GGGGTTGGGT GTTCTTCATC ATAACCTTTT ACACTGTCTT TTAAAATTGT ACACCTTTTT ATTGTACCAC
 68 W  A  T   H  A  C  V   P  T  D   P  N  P   Q  E  V  V   L  E  N   V  T  E   N  F  N  M   W  K  N   N  M  V

         ppu10I
         nsiI/avaIII                          ahaIII/draI                 draIII      ahaIII/draI
301 GAACAGATGC ATGAGGATAT AATCAGTTTA TGGGATCAAA GTTTAAAGCC ATGTGTAAAA TTAACCCCAC TCTGTGTTAC TTTAAATTGC ACTGATGCGG
    CTTGTCTACG TACTCCTATA TTAGTCAAAT ACCCTAGTTT CAAATTTCGG TACACATTTT AATTGGGGTG AGACACAATG AAATTTAACG TGACTACGCC
101 E  Q  M  H   E  D  I   I  S  L   W  D  Q  S   L  K  P   C  V  K   L  T  P  L   C  V  T   L  N  C   T  D  A  G

                                             gsuI/bpmI
401 GGAATACTAC TAATACCAAT AGTAGTAGCA GGGAAAAGCT GGAGAAAGGA GAAATAAAAA ACTGCTCTTT CAATATCACC ACAAGCGTGA GAGATAAGAT
    CCTTATGATG ATTATGGTTA TCATCATCGT CCCTTTTCGA CCTCTTTCCT CTTTATTTTT TGACGAGAAA GTTATAGTGG TGTTCGCACT CTCTATTCTA
135  N  T  T   N  T  N   S  S  S  R   E  K  L   E  K  G   E  I  K  N   C  S  F   N  I  T   T  S  V  R   D  K  M
                                  ^421,reverse

                                                                       scaI        scaI       scfI
501 GCAGAAAGAA ACTGCACTTT TTAATAAACT TGATATAGTA CCAATAGATG ATGATGATAG GAATAGTACT AGGAATAGTA CTAACTATAG GTTGATAAGT
    CGTCTTTCTT TGACGTGAAA AATTATTTGA ACTATATCAT GGTTATCTAC TACTACTATC CTTATCATGA TCCTTATCAT GATTGATATC CAACTATTCA
168 Q  K  E   T  A  L  F   N  K  L   D  I  V   P  I  D  D   D  D  R   N  S  T   R  N  S  T   N  Y  R   L  I  S
       ^43r2,reverse
```

```
                          stuI
                          haeI
601 TGTAACACCT CAGTCATTAC ACAGGCCTGT CCAAAGGTAT CATTTGAGCC AATTCCCATA CATTTCTGTA CCCCGGCTGG TTTTGCGCTT CTAAAGTGTA
    ACATTGTGGA GTCAGTAATG TGTCCGGACA GGTTTCCATA GTAAACTCGG TTAAGGGTAT GTAAAGACAT GGGGCCGACC AAAACGCGAA GATTTCACAT
201 C  N  T  S   V  I  T   Q  A  C   P  K  V  S   F  E  P   I  P  I   H  F  C  T   P  A  G   F  A  L   L  K  C  N

                                                                  bsp1407I        haeI
701 ATAATAAGAC GTTCAATGGA TCAGGACCAT GCAAAAATGT CAGCACAGTA CAATGTACAC ATGGAATTAG GCCAGTAGTA TCAACTCAAC TGCTGTTAAA
    TATTATTCTG CAAGTTACCT AGTCCTGGTA CGTTTTTACA GTCGTGTCAT GTTACATGTG TACCTTAATC CGGTCATCAT AGTTGAGTTG ACGACAATTT
235   N  K  T   F  N  G   S  G  P  C   K  N  V   S  T  V   Q  C  T  H   G  I  R   P  V  V   S  T  Q  L   L  L  N

                                      bstYI/xhoII                                                    pvuII                  aseI/asnI/
                                      bglII   apoI                                                   nspBII                  vspI
801 TGGCAGTCTA GCAGAAGGAG AGGTAGTAAT TAGATCTGAA AATTTCACGA ACAATGCTAA AACCATAATA GTACAGCTGA CAGAACCAGT AAAAATTAAT
    ACCGTCAGAT CGTCTTCCTC TCCATCATTA ATCTAGACTT TTAAAGTGCT TGTTACGATT TTGGTATTAT CATGTCGACT GTCTTGGTCA TTTTTAATTA
268  G  S  L   A  E  G  E   V  V  I   R  S  E   N  F  T  N   N  A  K   T  I  I   V  Q  L  T   E  P  V   K  I  N
                                                                                  ^f1,forward

                                      bst1107I
    bsp1407I                          accI  scfI
901 TGTACAAGAC CCAACAACAA TACAAGAAAA AGTATACCTA TAGGACCAGG GAGAGCATTT TATGCAACAG GAGACATAAT AGGAAATATA AGACAAGCAC
    ACATGTTCTG GGTTGTTGTT ATGTTCTTTT TCATATGGAT ATCCTGGTCC CTCTCGTAAA ATACGTTGTC CTCTGTATTA TCCTTTATAT TCTGTTCGTG
301 C  T  R  P   N  N  N   T  R  K   S  I  P  I   G  P  G   R  A  F   Y  A  T  G   D  I  I   G  N  I   R  Q  A  H
                                  ^875,reverse

                                                                                                                    eco81I
                                                                                                                    bsu36I/
                                                                                                                     mstII/
                                                                                                                     sauI
1001 ATTGTAACCT TAGTAGAACA GACTGGAATA ACACTTTAGG ACAGATAGTT GAAAAATTAA GAGAACAATT TGGGAATAAA ACAATAATCT TTAATCACTC
     TAACATTGGA ATCATCTTGT CTGACCTTAT TGTGAAATCC TGTCTATCAA CTTTTTAATT CTCTTGTTAA ACCCTTATTT TGTTATTAGA AATTAGTGAG
 335   C  N  L   S  R  T   D  W  N  N   T  L  G   Q  I  V   E  K  L  R   E  Q  F   G  N  K   T  I  I  F   N  H  S

           ppuMI
           eco0109I/draII                                                 apoI                                munI        scaI
1101 CTCAGGAGGG GACCCAGAAA TTGTAATGCA CAGTTTTAAT TGTAGAGGGG AATTTTTCTA CTGTAATACA ACACAATTGT TTGACAGTAC TTGGGATAAT
     GAGTCCTCCC CTGGGTCTTT AACATTACGT GTCAAAATTA ACATCTCCCC TTAAAAAGAT GACATTATGT TGTGTTAACA AACTGTCATG AACCCTATTA
 368  S  G  G   D  P  E  I   V  M  H   S  F  N   C  R  G  E   F  F  Y   C  N  T   T  Q  L  F   D  S  T   W  D  N

                                                                                                                nspI
                                                                                                                nspHI
                                  earI/ksp632I                                                                  aflIII
                                 eco57I
1201 ACTAAAGTGT CAAATGGCAC TAGCACTGAA GAGAATAGCA CAATCACACT CCCATGCAGA ATAAAGCAAA TTGTAAACAT GTGGCAGGAA GTAGGAAAAG
     TGATTTCACA GTTTACCGTG ATCGTGACTT CTCTTATCGT GTTAGTGTGA GGGTACGTCT TATTTCGTTT AACATTTGTA CACCGTCCTT CATCCTTTTC
 401 T  K  V  S   N  G  T   S  T  E   E  N  S  T   I  T  L   P  C  R   I  K  Q  I   V  N  M   W  Q  E   V  G  K  A
```

```
                                        mamI
                                        bsaBI        sspI                                                    bsaI
1301 CAATGTATGC CCCTCCCATC AGAGGACAAA TTAGATGTTC ATCAAATATT ACAGGGTTGC TATTAACAAG AGATGGAGGT AGTAACAACA GCATGAATGA
     GTTACATACG GGGAGGGTAG TCTCCTGTTT AATCTACAAG TAGTTTATAA TGTCCCAACG ATAATTGTTC TCTACCTCCA TCATTGTTGT CGTACTTACT
 435   M  Y  ^A   P  P  I   R  G  Q  I   R  C  S   S  N  I   T  G  L  L   L  T  R   D  G  G   S  N  N  S   M  N  E
            ^2,16.7f3,forward

                  gsuI/bpmI
        eco57I    ecoNI                    munI                                                              styI
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       ^c4rev4,reverse

                earI/ksp632I                                                styI
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 501 K  A  K  R   R  V  V   Q  R  E   K  R  A  V   G  I  G   A  V  F   L  G  F  L   G  A  A   G  S  T   M  G  A  A

                           haeI                                                                  alwNI
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                                  ^43f5,forward                                                  ^43r3,reverse

                                                                    eco81I        alwNI
                                        gsuI/bpmI                   bsu36I/mstII/sauI
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                                  styI  bsmI                    xbaI
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 601 G  K  L  I   C  T  T   S  V  P   W  N  A  S   W  S  N   K  S  L   D  K  I  W   D  N  M   T  W  M   E  W  E  R

                        hindIII
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     CTCTTTAACT CTTAATGTGT TCGAATTATA TGTGGAATTA ACTTCTTAGC GTCTTGGTTG TTCTTTTCTT ACTTGTTCTG AATAACCTTA ACCTAGTTAC
  635   E  I  E   N  Y  T   S  L  I  Y   T  L  I   E  E  S   Q  N  Q  Q   E  K  N   E  Q  D   L  L  E  L   D  Q  W
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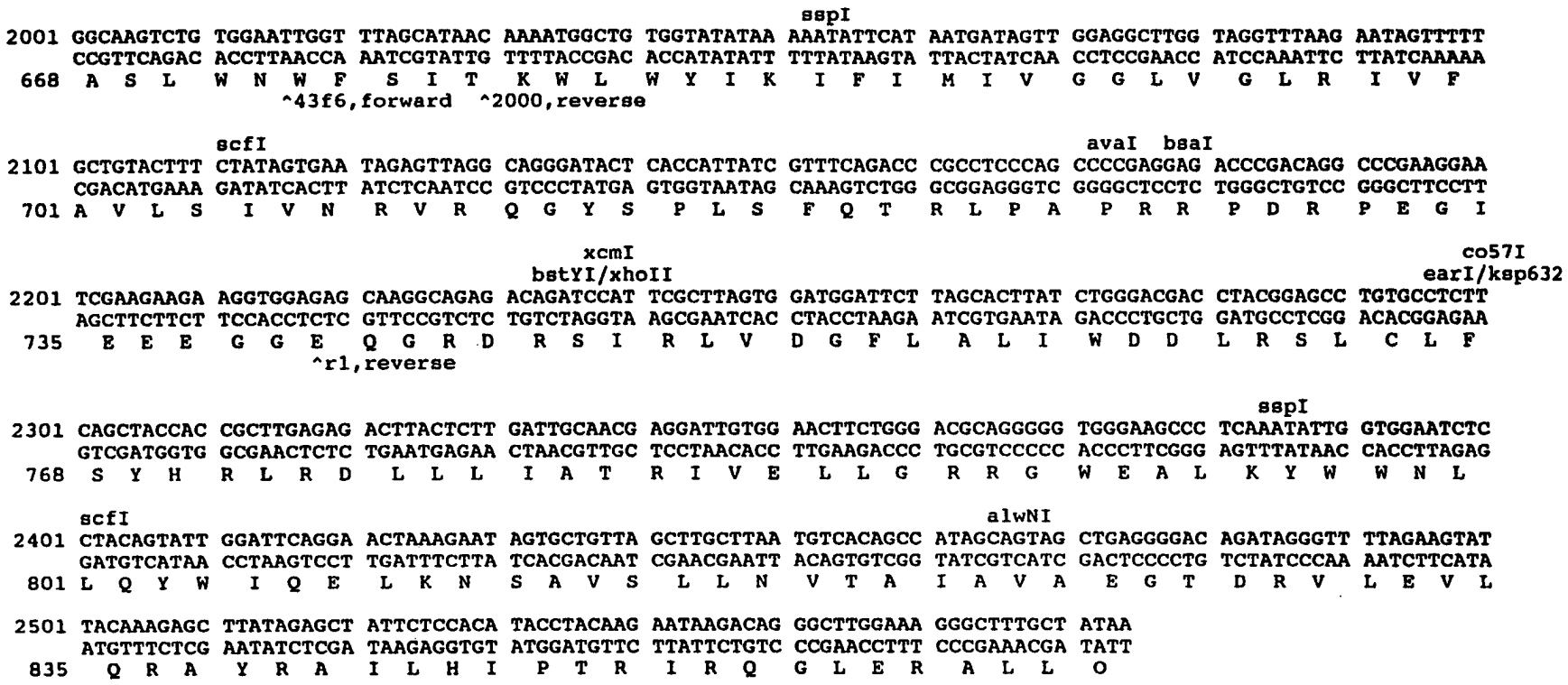

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668 A S L W N W F S I T K W L W Y I K I F I M I V G G L V G L R I V F

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CGACATGAAA GATATCACTT ATCTCAATCC GTCCCTATGA GTGGTAATAG CAAAGTCTGG GCGGAGGGTC GGGGCTCCTC TGGGCTGTCC GGGCTTCCTT
701 A V L S I V N R V R Q G Y S P L S F Q T R L P A P R R P D R P E G I

2201 TCGAAGAAGA AGGTGGAGAG CAAGGCAGAG ACAGATCCAT TCGCTTAGTG GATGGATTCT TAGCACTTAT CTGGGACGAC CTACGGAGCC TGTGCCTCTT
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2501 TACAAAGAGC TTATAGAGCT ATTCTCCACA TACCTACAAG AATAAGACAG GGCTTGGAAA GGGCTTTGCT ATAA
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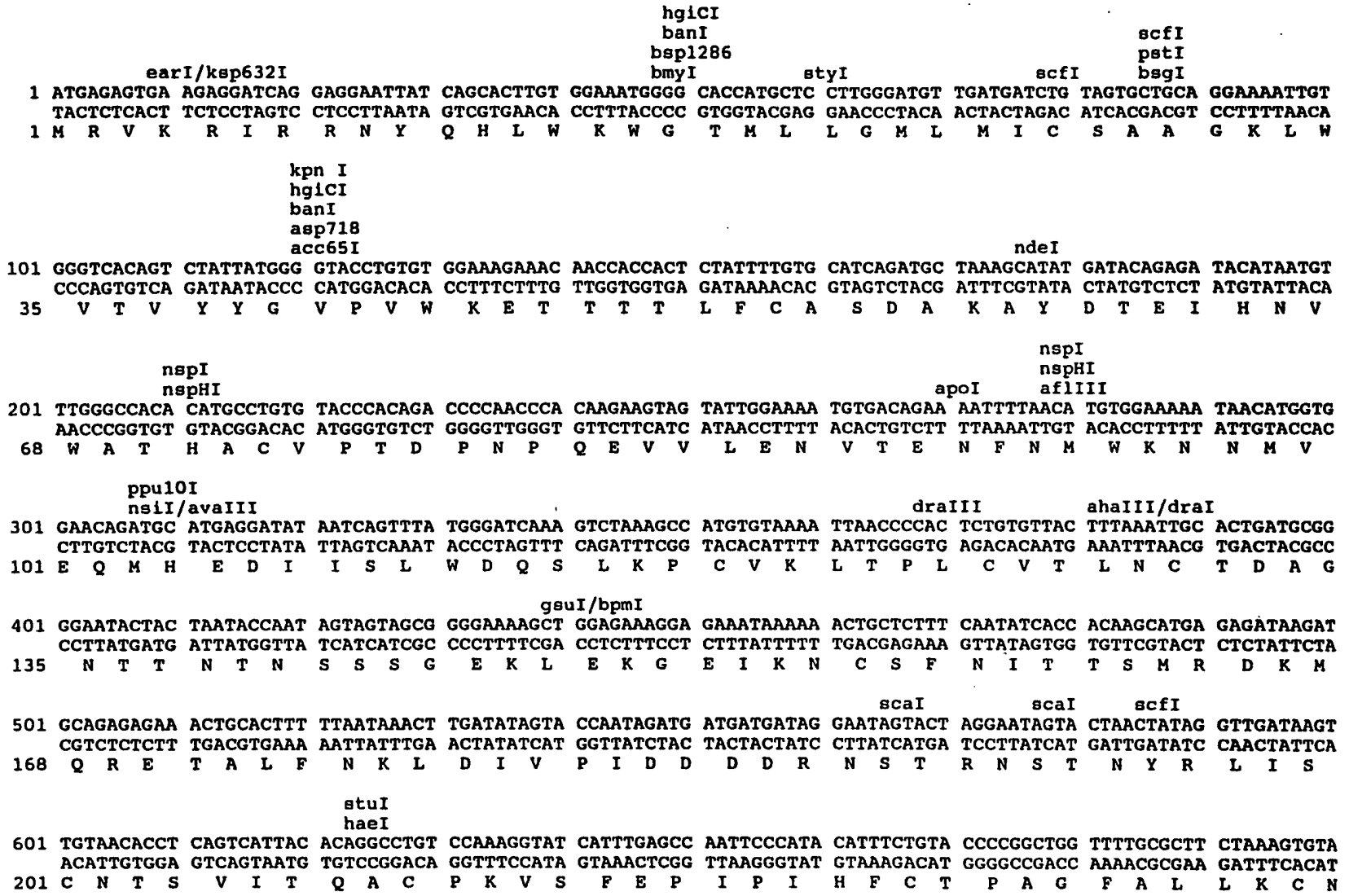
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styI
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kpn I
hgiCI
banI
asp718
acc65I
ndeI
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nspI
nspHI
nspI
nspHI
apoI
aflIII
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ppu10I
nsiI/avaIII
draIII
ahaIII/draI
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CTTGTCTACG TACTCCTATA TTAGTCAAAT ACCCTAGTTT CAGATTTCGG TACACATTTT AATTGGGGTG AGACACAATG AAATTTAACG TGACTACGCC
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gsuI/bpmI
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scaI
scaI
scfI
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168 Q R E T A L F N K L D I V P I D D D D R N S T R N S T N Y R L I S
stuI
haeI
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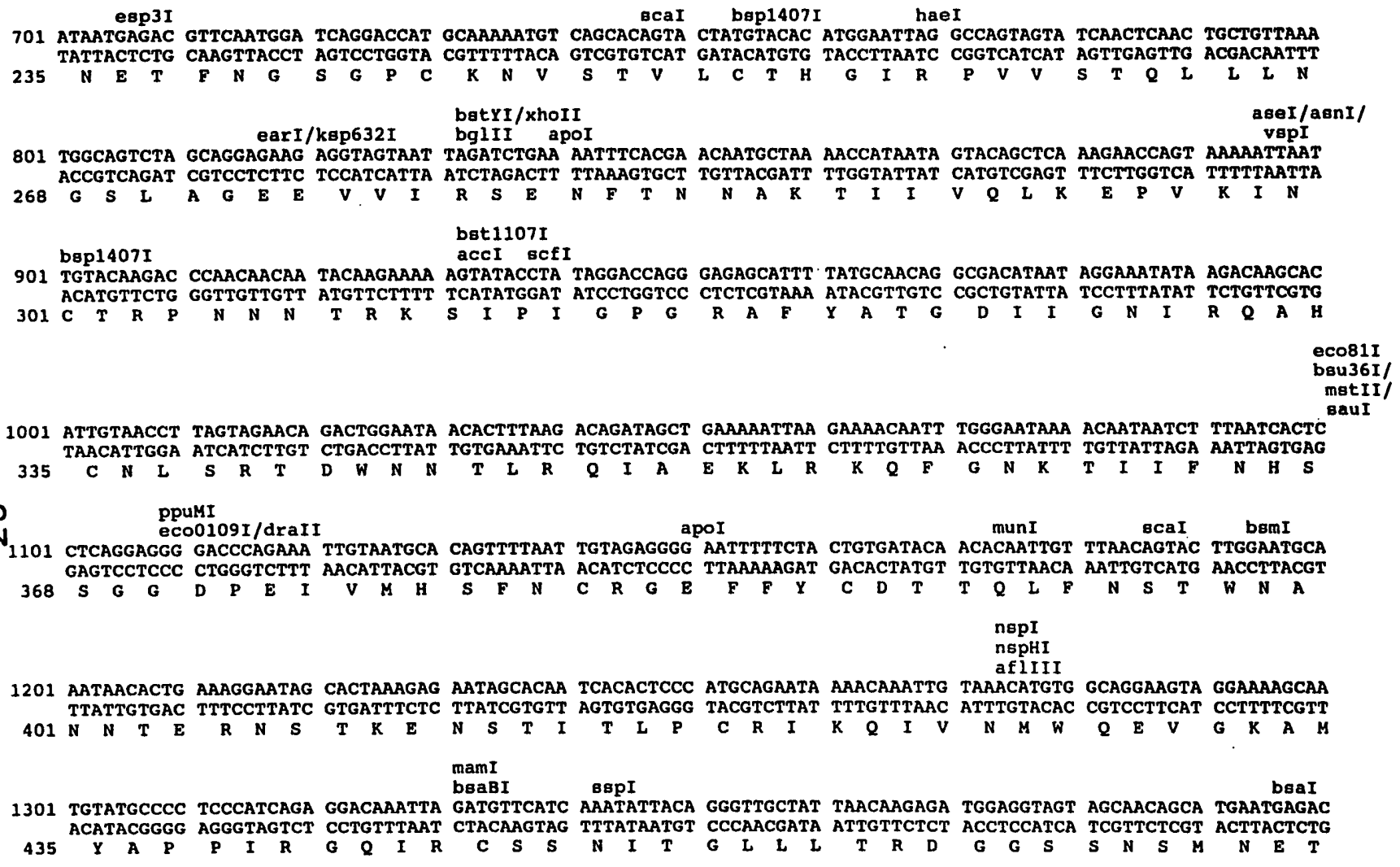

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 468  F  R  P   G  G  G  D   M  R  D   N  W  R   S  E  L  Y   K  Y  K   V  V  K   I  E  P  L   G  V  A   P  T  K

          earI/ksp632I                                          styI
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 501 A  M  R  R   V  V  Q   R  E  K   R  A  V  G   I  G  A   V  F  L   G  F  L  G   A  A  G   S  T  M   G  A  A  S

                    haeI                                                                  alwNI
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  535   I  T  L   T  V  Q   A  R  L  L   L  S  G   I  V  Q   Q  Q  N  N   L  L  R   A  I  E   A  Q  Q  H   L  L  Q

                                                        eco81I        alwNI
                                  gsuI/bpmI             bsu36I/mstII/sauI
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  568  L  T  V   W  G  I  K   Q  L  Q   A  R  V   L  A  V  E   R  Y  L   R  D  Q   Q  L  L  G   I  W  G   C  S  G

                                styI bsmI                       xbaI
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  601 K  L  I  C   T  T  S   V  P  W   N  A  S  W   S  N  K   S  L  D   K  I  W  D   N  M  T   W  M  E   W  E  R  E

                hindIII
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  635   I  E  N   Y  T  S   L  I  Y  T   L  I  E   E  S  Q   N  Q  Q  E   K  N  K   Q  D  L   L  E  L  D   Q  O  A

                                                  sspI
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  668  S  L  W   N  W  F  S   I  T  K   W  L  W   Y  I  K  I   F  I  M   I  V  G   G  L  V  G   L  R  I   V  F  A

                                                                                ppuMI
          scfI                                                                  avaI   eco0109I/draII
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  701 V  L  S  I   V  N  R   V  R  Q   G  Y  S  P   L  S  F   Q  T  R   L  P  A  P   R  G  P   D  R  P   K  G  I  E
```

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                                                   xcmI                                                                   eco57I
                                              bstYI/xhoII                                                           earI/ksp632I
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      TTCTTCTTCC ACCTCTCGTT CTGTCCCTGT CTAGGTAAGC GAATCACCTA CCTAAGAATC GTGAATAGAC CCTGCTAGAT GCCTCGGACA CGGAGAAGTC
 735   E  E  G   G  E  Q   D  R  D  R   S  I  R   L  V  D   G  F  L  A   L  I  W   D  D  L   R  S  L  C   L  F  S

                                                                                                     sspI         scfI
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                                                                   alwNI                  xbaI
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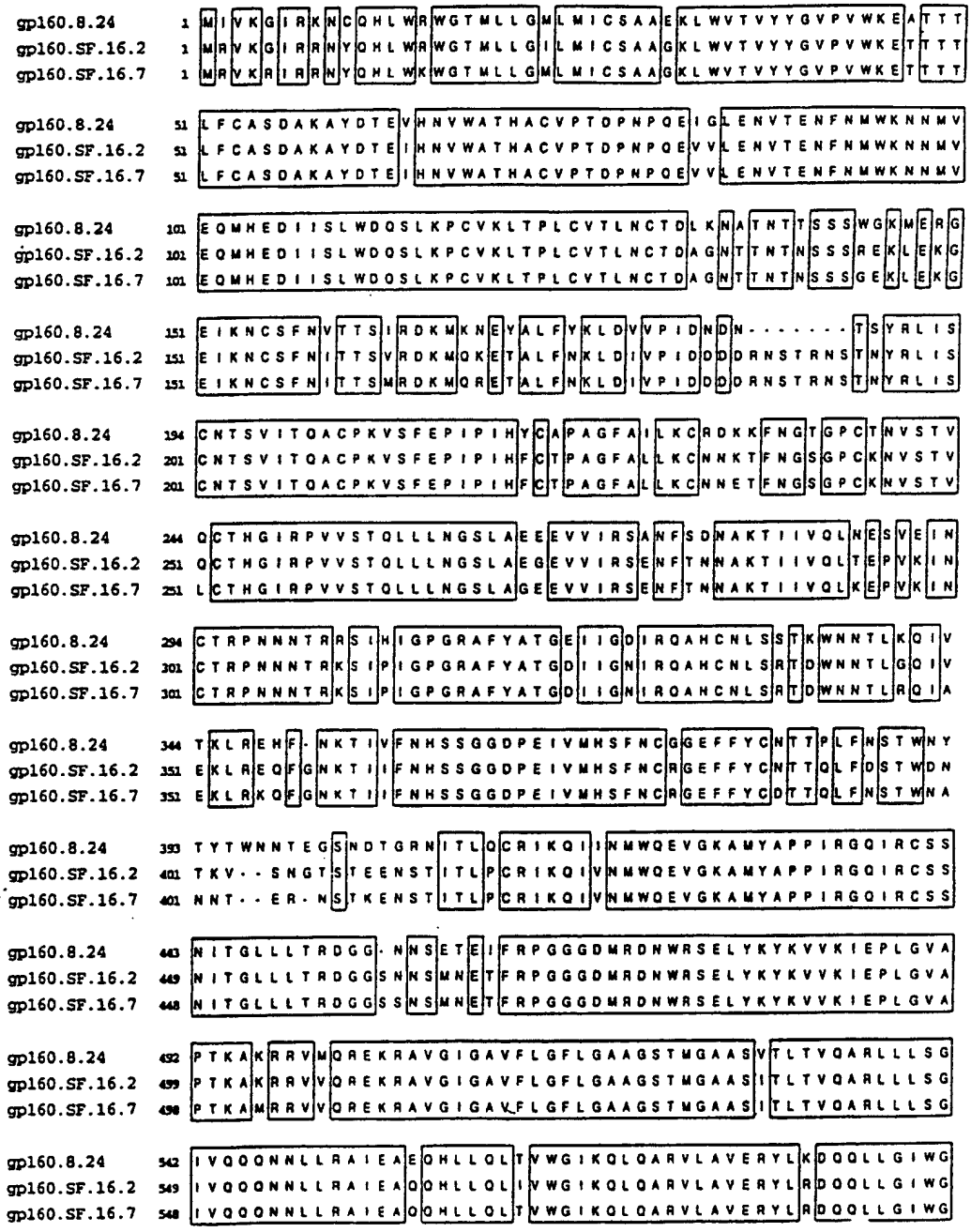
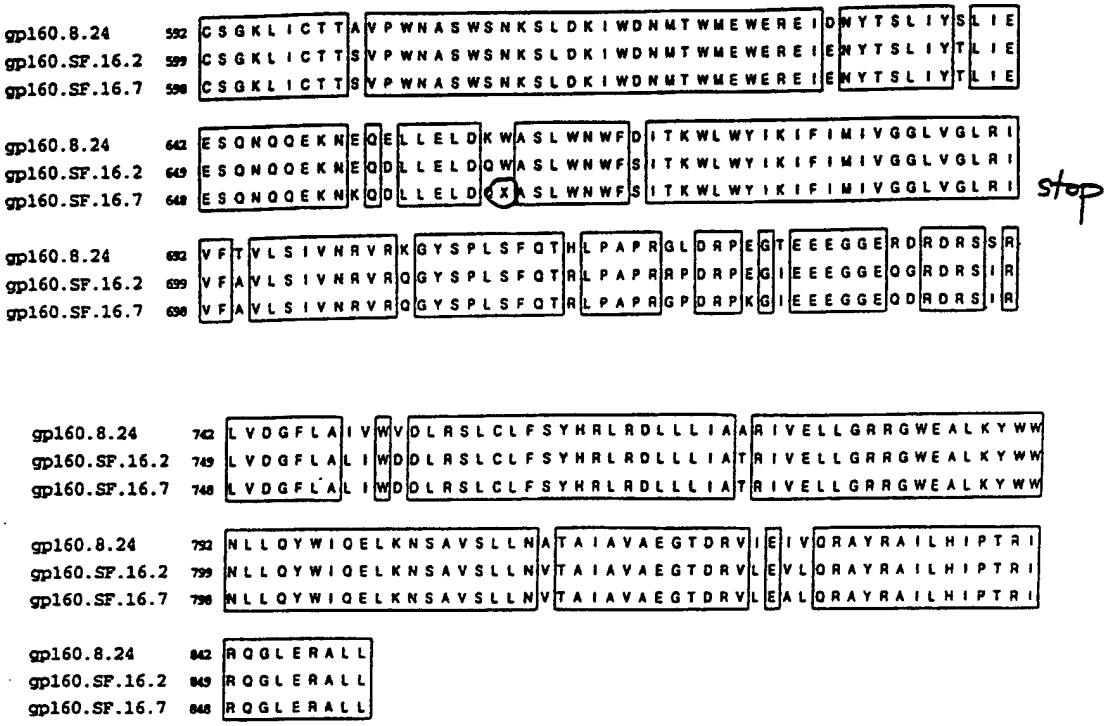
 801  Q  Y  W  I   Q  E  L   K  N  S   A  V  S  L   L  N  V   T  A  I   A  V  A  E   G  T  D   R  V  L   E  A  L  Q

2501  AAAGAGCTTA TAGAGCTATT CTCCACATAC CTACAAGAAT AAGACAAGGC TTGGAAAGGG CTTTGCTATA A
      TTTCTCGAAT ATCTCGATAA GAGGTGTATG GATGTTCTTA TTCTGTTCCG AACCTTTCCC GAAACGATAT T
 835   R  A  Y   R  A  I   L  H  I  P   T  R  I   R  Q  G   L  E  R  A   L  L  O
```

Table 3 illustrates the amino acid sequences for the MN, GNE_8, and GNE_{16} gp120 proteins. The regions of the sequences having identical amino acid sequences are enclosed in boxes.

TABLE 3

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gp160.SF.16.2 599 CSGKLICTTSVPWNASWSNKSLDKIWDNMTWMEWEREIENYTSLIYTLIE
gp160.SF.16.7 598 CSGKLICTTSVPWNASWSNKSLDKIWDNMTWMEWEREIENYTSLIYTLIE
gp160.8.24 642 ESQNQQEKNEQELLELDKWASLWNWFDITKWLWYIKIFIMIVGGLVGLRI
gp160.SF.16.2 649 ESQNQQEKNEQDLLELDQWASLWNWFSITKWLWYIKIFIMIVGGLVGLRI
gp160.SF.16.7 648 ESQNQQEKNKQDLLELDQXASLWNWFSITKWLWYIKIFIMIVGGLVGLRI
stop
gp160.8.24 692 VFTVLSIVNRVRKGYSPLSFQTHLPAPRGLDRPEGTEEEGGERDRDRSSR
gp160.SF.16.2 699 VFAVLSIVNRVRQGYSPLSFQTRLPAPRRPDRPEGIEEEGGEQGRDRSIR
gp160.SF.16.7 698 VFAVLSIVNRVRQGYSPLSFQTRLPAPRGPDRPKGIEEEGGEQDRDRSIR
gp160.8.24 742 LVDGFLAIVWVDLRSLCLFSYHRLRDLLLIAARIVELLGRRGWEALKYWW
gp160.SF.16.2 749 LVDGFLALIWDDLRSLCLFSYHRLRDLLLIATRIVELLGRRGWEALKYWW
gp160.SF.16.7 748 LVDGFLALIWDDLRSLCLFSYHRLRDLLLIATRIVELLGRRGWEALKYWW
gp160.8.24 792 NLLQYWIQELKNSAVSLLNATAIAVAEGTDRVIEIVQRAYRAILHIPTRI
gp160.SF.16.2 799 NLLQYWIQELKNSAVSLLNVTAIAVAEGTDRVLEVLQRAYRAILHIPTRI
gp160.SF.16.7 798 NLLQYWIQELKNSAVSLLNVTAIAVAEGTDRVLEALQRAYRAILHIPTRI
gp160.8.24 842 RQGLERALL
gp160.SF.16.2 849 RQGLERALL
gp160.SF.16.7 848 RQGLERALL

Nucleic acid sequences encoding gp120 from GNE_8 and GNE_{16} capable of expressing gp120 can be prepared by conventional means. The nucleotide sequence can be synthesized. Alternatively, another HIV nucleic acid sequence encoding gp120 can be used as a backbone and altered at any differing residues by site directed mutagenesis as described in detail in Example 1.

In a preferred embodiment, the nucleotide sequence is present in an expression construct containing DNA encoding gp120 under the transcriptional and translational control of a promoter for expression of the encoded protein. The promoter can be a eukaryotic promoter for expression in a mammalian cell. In cases where one wishes to expand the promoter or produce gp120 in a prokaryotic host, the promoter can be a prokaryotic promoter. Usually a strong promoter is employed to provide high level transcription and expression.

The expression construct can be part of a vector capable of stable extrachromosomal maintenance in an appropriate cellular host or may be integrated into host genomes. Normally, markers are provided with the expression construct which allow for selection of a host containing the construct. The marker can be on the same or a different DNA molecule, desirably, the same DNA molecule.

The expression construct can be joined to a replication system recognized by the intended host cell. Various replication systems include viral replication systems such as retroviruses, simian virus, bovine papilloma virus, or the like. In addition, the construct may be joined to an amplifiable gene, e.g. DHFR gene, so that multiple copies of the gp120 DNA can be made. Introduction of the construct into the host will vary depending on the construct and can be

achieved by any convenient means. A wide variety of prokaryotic and eukary tic hosts can be employed for expression of the proteins.

Preferably, the gp120 is expressed in mammalian cells that provide the same glycosylation and disulfide bonds as in native gp120. Expression of gp120 and fragments of gp120 in mammalian cells as fusion proteins incorporating N-terminal sequences of Herpes Simplex Virus Type 1 (HSV-1) glycoprotein D (gD-1) is described in Lasky, L. A. et al., 1986 (Neutralization of the AIDS retrovirus by antibodies to a recombinant envelope glycoprotein) Science 233: 209-212 and Haffar, O.K. et al., 1991 (The cytoplasmic tail of HIV-1 gp160 contains regions that associate with cellular membranes.) Virol. 180:439-441, respectively. A preferred method for expressing gp120 is described in Example 3. In the example, a heterologous signal sequence was used for convenient expression of the protein. However, the protein can also be expressed using the native signal sequence.

An isolated, purified GNE_8-gp120 and GNE_{16}-gp120 having the amino acid sequence illustrated in Tables 1-3 can be produced by conventional methods. For example, the proteins can be chemically synthesized. In a preferred embodiment, the proteins are expressed in mammalian cells using an expression construct of this invention. The expressed proteins can be purified by conventional means. A preferred purification procedure is described in Example 3.

gp120 Fragments

The present invention also provides gp120 fragments that are suitable for use in inducing antibodies for use in serotyping or in a vaccine formulation. A truncated gp120 sequence as used herein is a fragment of gp120 that is free from a portion of

the intact gp120 sequence beginning at either the amino or carboxy terminus of gp120. A truncated gp120 sequenc of this invention is free from the C5 domain. The C5 domain of gp120 is a major immunogenic site of the molecule. However, antibodies to the region do not neutralize virus. Therefore, elimination of this portion of gp120 from immunogens used to induce antibodies for serotyping is advantageous.

In another embodiment, the truncated gp120 sequence is additionally free from the carboxy terminus region through about amino acid residue 453 of the gp120 V5 domain. The portion of the V5 domain remaining in the sequence provides a convenient restriction site for preparation of expression constructs. However, a truncated gp120 sequence that is free from the entire gp120 V5 domain is also suitable for use in inducing antibodies.

In addition, portions of the carboxy terminus of gp120 can also be eliminated from the truncated gp120 sequence. The truncated gp120 sequence can additionally be free from the gp120 signal sequence. The truncated gp120 sequence can be free from the carboxy terminus through amino acid residue 111 of the gp120 C1 domain, eliminating most of the C1 domain but preserving a convenient restriction site. However, the portion of the C1 domain through the cysteine residue that forms a disulfide bond can additionally be removed, so that the truncated gp120 sequence is free from the carboxy terminus through amino acid residue 117 of the gp120 C1 domain. Alternatively, the truncated gp120 sequence can be free from the amino terminus of gp120 through residue 111 of the C1 domain, preserving the V2 disulfide bond. In a preferred embodiment, the truncated gp120 sequence is free from the amino terminus of gp120 through residue 111 of the

C1 domain and residue 453 through the carboxy terminus of gp120.

The truncated gp120 sequences can be produced by recombinant engineering, as described previously. Conveniently, DNA encoding the truncated gp120 sequence is joined to a heterologous DNA sequence encoding a signal sequence.

Serotyping method

The present invention also provides an improved serotyping method for HIV strains. The method comprises determining the serotypes of the V2, V3, and C4 domains of gp120.

HIV isolates can be serotyped by conventional immunoassay methods employing antibodies to the neutralizing epitopes in the V2, V3, and C4 domains for various strains of HIV. Preparation of the antibodies is described hereinbefore. The antibody affinity required for serotyping HIV using a particular immunoassay method does not differ from that required to detect other polypeptide analytes. The antibody composition can be polyclonal or monoclonal, preferably monoclonal.

A number of different types of immunoassays are well known using a variety of protocols and labels. The assay conditions and reagents may be any of a variety found in the prior art. The assay may be heterogeneous or homogeneous. Conveniently, an HIV isolate is adsorbed to a solid phase and detected with antibody specific for one strain of neutralizing epitope for each neutralizing epitope in the V2, V3, and C4 domain. Alternatively, supernatant or lysate from the cultured isolate which contains gp120 can be adsorbed to the solid phase. The virus or gp120 can be adsorbed by many well known non-specific binding methods. Alternatively, an anti-gp120 antibody,

preferably directed to the carboxy terminus of gp120 can be used to affix gp120 to the solid phase. A gp120 capture antibody and sandwich ELISA assay f r gp120 neutralizing epitopes is described by Moore, *AIDS Res. Hum. Retroviruses* 9:209-219 (1993). Binding between the antibodies and sample can be determined in a number of ways. Complex formation can be determined by use of soluble antibodies specific for the anti-gp120 antibody. The soluble antibodies can be labeled directly or can be detected using labeled second antibodies specific for the species of the soluble antibodies. Various labels include radionuclides, enzymes, fluorescers, colloidal metals or the like. Conveniently, the anti-gp120 antibodies will be labeled directly, conveniently with an enzyme.

Alternatively, other methods for determining the neutralizing epitopes can be used. For example, fluorescent-labeled antibodies for a neutralizing epitope can be combined with cells infected by the strain of HIV to be serotyped and analyzed by fluorescence activated cell sorting.

The serotype of the HIV isolate includes the strain of the neutralizing epitopes for the V2, V3, and C4 domains.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention. All literature citations herein are expressly incorporated by reference.

EXAMPLE 1

Identification of C4 Neutralizing Epitopes

The following reagents and methods were used in the studies described herein.

gp120 sequences and nomenclature. Amino acid residues are designated using the standard single letter code. The location of amino acids within the gp120 protein is specified using the initiator methionine residue as position 1. The designation LAI is used to describe the virus isolate from which the HIV-1_{BH10}, HIV-1_{IIIB}, HIV-1_{SRU}, HIV-1_{HXB2}, HIV-1_{HXB3} and HIV-1_{HXB10} substrains (molecular clones) of HIV-1 were obtained. The sequence of gp120 from IIIB substrain of HIV-1_{LAI} is that determined by Muesing *et al.* (30).

The sequence of gp120 from MN strain of HIV-1 is given with reference to the MNgp120 clone (MN_{GNE}). The sequence of this clone differs by approximately 2% from that of the MN_{1984} clone described by Gurgo *et al.* (13). The sequences of gp120 from the NY-5, JRcsf, Z6, Z321, and HXB2 strains of HIV-1 are those listed by Myers *et al.* (32) except where noted otherwise. The sequence of the Thai isolate A244 is that provided by McCutchan *et al.* (24). The variable (V) domains and conserved (C) domains of gp120 are specified according to the nomenclature of Modrow *et al.* (28).

Monoclonal antibody production and screening assays. Hybridomas producing monoclonal antibodies to MN-rgp120 (recombinantly produced gp120 from the MN strain of HIV) (3) were prepared and screened for CD4 blocking activity as described previously (7, 33). The binding of monoclonal antibodies to MN-rgp120 and to rgp120s from the IIIB, NY-5, Z6, Z321, JRcsf, and A244 strains of HIV-1 was assessed by enzyme linked

immunoadsorbant assays (ELISA) as described previously (33).

Virus binding and neutralization assays. The ability of monoclonal antibodies to neutralize HIV-1 infectivity *in vitro* was assessed in a colorimetric MT-2 cell cytotoxicity assay similar to that described previously (35). MT-2 cells and H9/HTLV-III_{MN} cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: contributed by Drs. Douglas Richman and Robert Gallo, respectively. Briefly, serial dilutions of antibody or serum were prepared in 50 μl volumes of complete and then 50 μl of a prediluted HIV-1 stock was added to each well. After incubation for 1 hr at 4°C, 100 μl of a 4 x 10^5 MT-2 cell/ml suspension was added. After incubation of the plates for 5 days at 37°C in 5% CO_2, viable cells were measured using metabolic conversion of the formazan MTT dye. Each well received 20 μl of a 5 mg/ml MTT solution in PBS.

After a 4 hr incubation at 37°C, the dye precipitate was dissolved by removing 100 μl of the cell supernatant, adding 130 μl of 10% Triton X-100 in acid isopropanol, then pipeting until the precipitate was dissolved. The optical density of the wells was determined at 540 nm. The percentage inhibition was calculated using the formula:

$$\frac{1-(\text{virus control}-\text{experimental})}{(\text{virus control}-\text{medium control})}$$

Cell surface staining of HIV-1 infected cells with monoclonal antibodies. H9 cells (2 x 10^5) chronically infected with the IIIB, HXB2, HXB3, and HX10 substrains of HIV-1_{LAI} or with HIV-1_{MN} were incubated for 30 min at room temperature with monoclonal antibodies (10 μg per ml) in 100 μl of RPMI 1640 cell culture media

containing 1% FCS. Cells were washed and then incubated with 20 μg per ml of fluorescein-conjugated, affinity-purified, goat antibody to mouse IgG $(Fab')_2$ (Cappel, West Chester, PA) for 30 min. Cells were washed, fixed with 1% paraformaldehyde and the bound antibody was quantitated by flow cytometry using a FACSCAN (Becton-Dickenson, Fullerton, CA).

Fluorescence data was expressed as percentage of fluorescent cells compared to the fluorescence obtained with the second antibody alone. Fluorescence was measured as the mean intensity of the cells expressed as mean channel number plotted on a log scale.

Fragmentation of the MN-rgp120 gene. Fragments of the MN-rgp120 gene were generated using the polymerase chain reaction (PCR) (17). Briefly, forward 30-mer oligonucleotide DNA primers incorporating a Xho 1 site, and reverse 36-mer oligonucleotide DNA primers containing a stop codon followed by a Xba 1 site were synthesized and used for the polymerase chain reactions. Thirty cycles of the PCR reaction were performed using 0.3 μg of a plasmid containing the gene for gp120 from the MN strain of HIV-1 (pRKMN. D533) and 0.04 nM of a designated primers. The PCR reaction buffer consisted of 0.1 M Tris buffer (pH 8.4), 50 mM KCl, 0.2 mM 4dNTP (Pharmacia, Piscataway, NJ), 0.15 M $MgCl_2$ and 0.5 Unit of Taq Polymerase (Perkin-Elmer Cetus, Norwalk, CN) and a typical PCR cycle consisted of a 60 second denaturation step at 94°C, followed by a 45 second annealing step at 55° C, and then an extension step at 72° C for 45 seconds.

Following the PCR amplification, the PCR products were purified by phenol and chloroform extraction, and then ethanol precipitated. The purified products were then digested with the restriction endonucleases Xho1 and Xba1. The resulting PCR products were gel purified

using 1% agarose (SEAKEM, FMC Bioproducts, Rockland, ME) or 5% polyacrylamide gel electr ph resis (PAGE) and then isolated by electroelution.

Site directed mutagenesis of the MN-rgp120 C4 domain. A recombinant PCR technique (15) was utilized to introduce single amino acid substitutions at selected sites into a 600 bp Bgl II fragment of MN-rgp120 that contained the C4 domain. This method entailed the PCR amplification of overlapping regions of the C4 domain of gp120 using primers that incorporated the desired nucleotide changes. The resultant PCR products were then annealed and PCR amplified to generate the final product. For these reactions 18-mer "outside" primers encoding the wild type sequence (Bgl II sites) were amplified with 36-mer "inside" primers that contained the alanine or glutamic acid residue changes. The first PCR reaction included 1X of the Vent polymerase buffer (New England Biolabs, Beverly, MA), 0.2 mM of 4dNTP (Pharmacia, Piscataway, N.J.), 0.04 nM of each synthetic oligonucleotide, 0.3 μg of linearized plasmid, pRKMN.D533, which contained the MN-rgp120 gene. Thirty PCR cycles were performed consisting of the following sequence of steps: 45 seconds of denaturation at 94·C, 45 second of annealing at 55°C and 45 seconds of extension at 72°C. Following PCR amplification, the product pairs were gel purified using a 1% solution of low melt agarose (SeaPlaque, FMC Bioproducts, Rockland, ME).

The agarose containing PCR product was melted at 65°C and combined with the PCR product of the overlapping pair and equilibrated to 37°C. Added to this (20 μl) was 10 μl of 10X Vent Polymerase buffer, 10 μl of 2 mM 4dNTP, 0.04 nM each of the "outside" wild type 18 mer oligonucleotides, 57 μl of H_2O and 1 unit of

Vent Polymerase. Thirty PCR cycles were performed as previously above.

The resulting PCR products were purified and digested with the Bgl II endonuclease. The digested PCR product was then ligated into the mammalian cell expression vector pRKMN.D533, which had been digested with Bgl II allowing for the removal of a 600 bp fragment. Colonies containing the correct insertion were identified and Sequenase 2.0 supercoil sequencing was employed to check for fidelity and the incorporation of the desired mutation.

Expression of gp120 fragments in mammalian cells. Fragments of the MN and IIIB gp120 were expressed in mammalian cells as fusion proteins incorporating N-terminal sequences of Herpes Simplex Virus Type 1 (HSV-1) glycoprotein D (gD-1) as described previously (14, 22). Briefly, isolated DNA fragments generated by the PCR reaction were ligated into a plasmid (pRK.gD-1) designed to fuse the gp120 fragments, in frame, to the 5' sequences of the glycoprotein D (gD) gene of Type 1 Herpes Simplex Virus (gD-1)and the 3' end to translational stop codons. The fragment of the gD-1 gene encoded the signal sequence and 25 amino acids of the mature form of HSV-1 protein. To allow for expression in mammalian cells, chimeric genes fragments were cloned into the pRK5 expression plasmid (8) that contained a polylinker with cloning sites and translational stop codons located between a cytomegalovirus promotor and a simian virus 40 virus polyadenylation site.

The resulting plasmids were transfected into the 293s embryonic human kidney cell line (12) using a calcium phosphate technique (11). Growth conditioned cell culture media was collected 48 hr after transfection, and the soluble proteins were detected by

ELISA or by specific radioimmunoprecipitation where metabolically labeled proteins from cell culture supernatants were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography as described previously (1, 18).

Radioimmunoprecipitation of MN-rgp120 mutants. Plasmids directing the expression of the MN-rgp120 C4 domain mutants were transfected into 293s cells as described above. Twenty four hours following the transfection, the cells were metabolically labeled with [^{35}S]-labeled methionine or cysteine as described previously (1). The labeled cell culture supernatants were then harvested and 0.5 ml aliquots were reacted with 1-5 μg of the monoclonal antibody or with 2 μl of the polyclonal rabbit antisera to MN-rgp120 and immunoprecipitated with Pansorbin (CalBiochem, La Jolla, CA) as described previously (1). The resulting Pansorbin complex was pelleted by centrifugation, washed twice with a solution containing PBS, 1% NP-40 and 0.05% SDS and then boiled in a PAGE sample buffer containing 1% 2-mercaptoethanol. The processed samples were the analyzed by SDS-PAGE and visualized by autoradiography (1, 18).

Assays to measure the binding of monoclonal antibodies to mutagenized MN-rgp120 polypeptides. An ELISA was developed to screen for reactivity of MN-rgp120 fragments and mutant proteins with various monoclonal antibodies. In this assay, 96 well microtiter dishes (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight with mouse monoclonal antibody (5B6) to gD-1, at a concentration of 2.0 μg/ml in phosphate buffered saline (PBS). The plates were blocked in a PBS solution containing 0.5% bovine serum

albumin (PBSA) and then incubated with growth conditi ned cell cultur m dium fr m transfected cells expressing the recombinant gp120 variants for 2 hr at room temperature. The plates were washed three times in PBS containing 0.05% Tween 20 and then incubated with the purified, HRPO-conjugated monoclonal antibodies. Following a 1 hr incubation, the plates were washed three times and developed with the colorimetric substrate, o-phenylenediamine (Sigma, St. Louis, MO).

The optical densities in each well were then read in a microtiter plate reading spectrophotometer at 492 nm. Each cell culture supernatant containing fragments or mutated rgp120s was normalized for expression based on the titering of its reactivity to the V3 binding monoclonal antibody 1034 or to a rabbit polyclonal antisera to MN-rgp120. Data from these experiments were expressed as a ratio of the optical densities obtained with the CD4 blocking monoclonal antibodies binding to the fragments or MN-rgp120 mutants compared with the full length wild type rgp120s.

To normalize for different concentrations of MN-rgp120-derived protein in the cell culture supernatants, the binding of the CD4 blocking monoclonal antibodies to each preparation was compared to that of an HRPO-conjugated monoclonal antibody to the V3 domain of MN-rgp120 (1034). Data from these experiments were expressed as a ratio of the optical densities obtained with the CD4 blocking monoclonal antibodies to the HRPO conjugated V3 reactive monoclonal antibody.

CD4 binding assays. The ability of monoclonal antibodies to inhibit the binding of MN-rgp120 to recombinant soluble CD4 (rsCD4) was determined in a

solid phase radioimmunoassay similar to that described previously (33). The effect of single amino acid substitutions on the binding of MN-rgp120 mutants to CD4 was determined in a co-immunoprecipitation assay similar to that described previously (21). Briefly, 293 cells were metabolically labeled with ^{35}S-methionine 24 hr after transfection with plasmids expressing MN-rgp120 variants. Growth conditioned cell culture medium (0.5 ml) was then incubated with 5.0 μg of recombinant sCD4 for 90 minutes at room temperature. Following this incubation, 5.0 μg of an anti-CD4 monoclonal antibody (465), known to bind to an epitope remote from the gp120 binding site, was added and allowed to incubate another 90 minutes at room temperature.

The gp120-CD4-antibody complexes were precipitated with Pansorbin that had been washed with PBS, preabsorbed with 0.1% bovine serum albumin and then bound with 50 μg of an affinity purified rabbit anti-mouse IgG (Cappel, West Chester, PA). The pellet was washed twice with PBS 1% NP-40, 0.05% SDS, and then boiled in beta mercaptoethanol containing SDS-PAGE sample buffer. The immunoprecipitation products were resolved by SDS PAGE and visualized by autoradiography as described previously (1, 21).

Antibody affinity measurements. Anti-gp120 antibodies were iodinated with Na ^{125}I with iodogen® (Pierce, Rockford, IL). Briefly, 50 μg of antibody in PBS was placed in 1.5 ml polypropylene microcentrifuge tubes coated with 50 μg of Iodogen. Two millicuries of carrier free Na[^{125}I] was added. After 15 min., free ^{125}I was separated from the labeled protein by chromatography on a PD-10 column (Pierce, Rockford, IL) pre-equilibrated in PBS containing 0.5% gelatin.

Antibody concentrations following iodination were determined by ELISA to calculate specific activities.

For binding assays, 96-well microtiter plates were coated with 100 µl/well of a 10 µg/ml solution of MN-rgp120 or IIIBrgp120 in 0.1 M bicarbonate buffer, pH 9.6 and incubated for 2 hr at room temperature or overnight at 4°C. To prevent non-specific binding, plates were blocked for 1-2 hr at room temperature with 200 µl/well of a gelatin solution consisting of PBS containing 0.5% (wt/vol) gelatin and 0.02% sodium azide. Unlabeled anti-gp120 monoclonal antibody (0 to 400 nM) was titrated (in duplicate) in situ and radiolabeled antibody was added to each well at a concentration of 0.5 nM.

After a 1-2 hr incubation at room temperature, the plate was washed 10x with the PBS/0.5% gelatin/0.02% azide buffer to remove free antibody. The antibody-gp120 complexes were solubilized with 0.1 N NaOH/0.1% SDS solution and counted in a gamma counter. The data were analyzed by the method of Scatchard (40) using the Ligand analytical software program (31). K_d values reported represent the means of four independent determinations.

RESULTS

Characterization of monoclonal antibodies to MN-rgp120 that block CD4 binding. Monoclonal antibodies prepared from mice immunized with MN-rgp120 (3, 33), were screened for the ability to bind to MN-rgp120 coated microtiter dishes by ELISA as described previously (33). Of the thirty five clones obtained, seven were identified (1024, 1093, 1096, 1097, 1110, 1112, and 1127) that were able to inhibit the binding of MN-rgp120 to recombinant CD4 in ELISA (Figure 1) or solid phase or cell surface radioimmunoassays (21, 33). Previous studies have shown that two distinct classes

of CD4 blocking monoclonal antibodies occur: those that bind to conformation dependent (discontinuous) epitopes (16, 26, 33, 35, 45) and those that bind to conformation independent (sequential) epitopes (4, 7, 21, 33, 43).

To distinguish between these two alternatives, the binding of the monoclonal antibodies to denatured (reduced and carboxymethylated) MN-rgp120 (RCM-gp120) was measured by ELISA as described previously (33). As illustrated in Table 4, below, it was found that all of the CD4 blocking monoclonal antibodies reacted with the chemically denatured protein; indicating that they all recognized conformation independent (sequential) epitopes.

Table 4

Properties of monoclonal antibodies t MN-rgp120

MAb	CD4 Inhibitors	HIV-1 mn Neutralization	HIV-1 mn V3	CM-rgp120	C4 Domain peptides	rg120 cross reactivity
1024	+	+	-	+	-	2
1093	+	+	-	+	-	2
1096	+	+	-	+	-	2
1097	+	+	-	+	-	2
1110	+	+	-	+	-	2
1112	+	+	-	+	-	2
1127	+	+	-	+	-	2
1026	-	+	+	+	-	1,2,3,4,6
1092	-	-	-	+	-	1,2,3,4,5
1126	-	-	-	+	-	1,2,3,5,7
1086	-	-	-	+	-	2
13H8	+	-	-	+	1,3	1,2,3,4,5,6,7

rgp120 cross reactivity: 1, IIIB-rg120; 2, MN-rgp120, 3, NYS-rgp120; 4, JrCSF-rgp120; 5, Z6-rgp120; 6, Z321-rgp120; 7, A244-rgp120

C4 domain peptides:
1, FINMWQEVGKAMYAPPIS (SEQ. ID. NO. 24);
2, MWQEVGKAMYAP (SEQ. ID. NO. 25) ;
3, GKAMYAPPIKGQIR (SEQ. ID. NO. 26)

The cross reactivity of these monoclonal antibodies was assessed by ELISA as described previously (33). In these experiments, the ability of the monoclonal antibodies to bind to a panel of seven rgp120s, prepared from the IIIB, MN, Z6, Z321, NY-5, A244, and JRcsf isolates of HIV-1, was measured by ELISA (33). It was found that all of the CD4 blocking monoclonal antibodies were strain specific and bound only to gp120 from the MN strain of HIV-1 (Table 4). However, other antibodies from the same fusion

(1026,1092, and 1126) exhibited much broader cross reactivity (Table 4, Figure 2), as did a CD4 blocking monoclonal antibody to IIIB-rgp120 (13H8) described previously (33).

Further studies were performed to characterize the neutralizing activity of the antibodies to MN-rgp120. In these studies, monoclonal antibodies were incubated with cell free virus (HIV-1_{MN}), and the resulting mixture was then used to infect MT-2 cells in microtiter plates. After 5 days, the plates were developed by addition of the colorimetric dye, MTT, and cell viability was measured spectrophotometrically. It was found (Table 4, Figure 2) that all of the CD4 blocking monoclonal antibodies were able to inhibit viral infectivity. However the potency of the monoclonal antibodies varied considerably with some monoclonal antibodies (eg. 1024) able to inhibit infection at very low concentrations (IC_{50} of 0.08 μg per ml) whereas other monoclonal antibodies (eg. 1112) required much higher concentrations (IC_{50} of 30 μg per ml). In control experiments two monoclonal antibodies to MN-rgp120 from the same fusion (eg.1086,1092) were ineffective, whereas the 1026 monoclonal antibody exhibited potent neutralizing activity. Similarly, monoclonal antibodies to the V3 domain of IIIB-rgp120 (10F6, 11G5) known to neutralize the infectivity HIV-1_{IIIB} (33), were unable to neutralize the HIV-1_{MN} virus.

Binding studies using synthetic peptides were then performed to further localize the epitopes recognized by these monoclonal antibodies as described previously (33). When a peptide corresponding to the V3 domain (3) of MN-rgp120 was tested, it was found that none of the CD4 blocking antibodies showed any reactivity. However the epitope recognized by the non-CD4 blocking monoclonal antibody, 1026, prepared against MN-rgp120 could be localized to the V3 domain by virtue of its

binding to this peptide. In other experiments, three synthetic peptides from the C4 domain of gp120 that incorporated sequences recognized by the CD4 blocking, weakly neutralizing monoclonal antibodies described by McKeating et al. (26) were tested (Table 4). It was found that none of the CD4 blocking monoclonal antibodies to MN-rgp120 reacted with these peptides, however the non-neutralizing, CD4 blocking 13H8 monoclonal antibody bound to the peptides corresponding to residues 423-440 of IIIB-gp120 and residues 431-441 of MN-gp120, but not to that corresponding to residues 426-437 of IIIB-gp120. Thus the 13H8 monoclonal antibody recognized a epitope that was similar, if not identical, to that described by McKeating et al. (26). This result is consistent with the observation that the 13H8 monoclonal antibody and the monoclonal antibodies described by Cordell et al. (4) and McKeating et al. (26) exhibited considerable cross reactivity, whereas the antibodies to MN-rgp120 were highly strain specific.

CD4 blocking antibodies recognize epitopes in the C4 domain. Previously, a strain specific, CD4 blocking monoclonal antibody (5C2) raised against IIIB-rgp120 was found to recognize an epitope in the C4 domain of IIIB-rgp120 (21, 33). Although the 5C2 monoclonal antibody was able to block the binding of rgp120 to CD4, it was unable to neutralize HIV-1 infectivity in vitro (7). Affinity columns prepared from 5C2 adsorbed an 11 amino acid peptide (residues 422 to 432) from a tryptic digest of gp120 (21), however monoclonal antibody 5C2 was unable to recognize this peptide coated onto wells of microtiter dishes in an ELISA format (Nakamura et al., unpublished results).

To determine whether the CD4 blocking monoclonal antibodies raised against MN-rgp120 recognized the

corresponding epitope in the C4 domain of MN-rgp120, a series of overlapping fragments, spanning the V4 and C4 domains of HIV-1_{MN} gp120, were prepared for expression in mammalian cells. A diagram of the fragments expressed is shown in Figures 3A and 3B. The C4 domain fragments were expressed as fusion proteins that incorporated the signal sequence and amino terminal 25 amino acids of HSV-1 glycoprotein D as described above.

Plasmids directing the expression of the chimeric C4 domain fragments were transfected into 293 cells, and their expression was monitored by radioimmunoprecipitation studies where a monoclonal antibody, 5B6, specific for the mature amino terminus of glycoprotein D was utilized. It was found (Figure 3B) that all of the fragments were expressed and exhibited mobilities on SDS-PAGE gels appropriate for their size. Thus fMN.368-408 (lane 1) exhibited a mobility of 19 kD; fMN.368-451 (lane 2) exhibited a mobility of 29 kD; fMN.419-433 (lane 3) exhibited a mobility of 6 kD, and fMN.414-451 (lane 4) exhibited a mobility of 6.1 kD.

The binding of monoclonal antibody 1024 to the recombinant fragments was then determined by ELISA (as described in Example 1). It was found (Figure 3A) that monoclonal antibody 1024 reacted with the fragments that contained the entire C4 domain of MN-rgp120 ($fMN_{368-451}$, $fMN_{404-455}$), but failed to bind to a fragment derived from the adjacent V4 domain ($fMN_{368-408}$) or to another fragment that contained V4 domain sequences and the amino terminal half of the C4 domain ($fMN_{368-428}$). The fact that 1024 bound to the $fMN_{414-451}$ and $fMN_{419-443}$ fragments demonstrated that the epitopes recognized by all of these monoclonal antibodies were contained entirely between residues 419 and 443 in the C4 domain.

Residues recognized by monoclonal antibodies that block binding of MN-rgp120 to CD4. To identify specific amino acid residues that might be part of the epitopes recognized by these monoclonal antibodies, the sequence of the C4 domain of MN-rgp120 was compared to those of the gp120s from the six other rgp120s that failed to react with the CD4 blocking monoclonal antibodies (Figure 4). It was noted that the sequence of MN-rgp120 was unique in that K occurred at position 429 whereas the other rgp120s possessed either E,G, or R at this position. Another difference was noted at position 440 where E replaced K or S. To evaluate the significance of these substitutions, a series of point mutations were introduced into the MN-rgp120 gene (Figure 5). Plasmids expressing the mutant proteins were transfected into 293s cells, and expression was verified by radioimmunoprecipitation with a monoclonal antibody (1034) directed to the V3 domain of MN-rgp120. Cell culture supernatants were harvested and used for the monoclonal antibody binding studies shown in Table 6. To verify expression, radio-immunoprecipitation studies using cell culture supernatants from cells metabolically labeled with [^{35}]S-methionine were performed using the 1024 monoclonal antibody specific for the C4 domain of MN-rgp120 (A) or the 1034 monoclonal antibody specific for the V3 domain of MN-rgp120. Immune complexes were precipitated with the use of fixed S. aureus and the adsorbed proteins were resolved by SDS-PAGE. Proteins were visualized by autoradiography. The samples were: Lane 1, MN.419A; lane 2 MN.421A; lane 3 MN.429E; lane 4, MN.429A; lane 5, MN.432A; lane 6, MN.440A; lane 7, MN-rgp120. The immunoprecipitation study showed that 1024 antibody binds well to all the variants except 3 and 4 which are mutated at residue 429. 1034 antibody

was used as a control and precipitates with anti-V3 antibodies.

The effect of these mutations on the binding of the CD4 blocking monoclonal antibodies was then evaluated by ELISA as illustrated in Table 5, below.

Table 5

Binding of CD4 blocking monoclonal antibodies to C4 domain mutants

Proteins/ MAbs	1024	1093	1096	1097	1110	1112	1127	5C2
MN-rgp120	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.05
MN-419A	1.11	1.10	0.94	1.21	0.78	0.95	1.10	ND
MN-421A	1.11	1.60	0.88	1.42	1.34	0.91	1.10	ND
MN-429E	0.03	0.07	0.11	0.04	0.10	0.10	0.02	ND
MN-429A	0.10	0.07	0.14	0.04	0.09	0.11	0.05	ND
MN-432A	0.77	0.15	0.59	0.08	0.12	0.24	0.26	ND
MN-440A	1.06	1.13	1.08	0.87	1.12	1.0	1.3	ND
IIIB-rgp120	0.03	ND	ND	ND	ND	ND	ND	1.0
MN-423F	ND	ND	ND	ND	ND	ND	ND	0.45
MN-423F,429E	ND	ND	ND	ND	ND	ND	ND	1.09

Data represent the relative binding of MAbs to the native and mutant forms of rgp120. Values were calculated by dividing the binding (determined by ELISA) of the CD4 blocking MAbs to the proteins indicated by the values obtained for the binding of a V3 specific MAb (1034) to the same proteins (as described in Example 1).

It was found that replacement of K_{440} with an A residue (MN.440A) had no effect on the binding of the 1024 monoclonal antibody or any of the other CD4 blocking monoclonal antibodies (Table 5). The significance of K at position 429 was then evaluated by substitution of either A (MN.429A) or E (MN.429E) at this location. It was found that the A for K substitution at position 429 (MN.420A) markedly reduced the binding of the 1024 monoclonal antibody and all of the other CD4 blocking monoclonal antibodies (Table 5).

Similarly, the replacement of E for K (MN.429E) at this position totally abrogated the binding of the 1024 monoclonal antibody and all of the other CD4 blocking monoclonal antibodies (Table 5). Several other mutants were constructed to evaluate the role of positively charged residues in the C4 domain. It was found that A for K substitutions at positions 419 (MN.419A) and 421(MN.421A) failed to interfere with the binding of any of the CD4 blocking monoclonal antibodies as illustrated in Table 6, below.

Table 6

Correlation Between Antibody Binding Affinity and Virus Neutralizing Activity

MAb	Block	K_d, nM[c]	IC_{50}, nM[d]
1024[e]	+	2.7 ± 0.9	0.4
1086[e,f]	-	9.7 ± 2.2	-
1093[e]	+	9.9 ± 2.6	3.3
1096[e]	+	10 ± 6	12
1097[e]	+	13.4 ± 3.7	12
1110[e]	+	12.1 ± 1.7	12
1112[e]	+	20 ± 4.4	200
1127[e]	+	9.3 ± 4	3.3
1086[e,f]	-	9.7 ± 2.2	-
13H8[f,g]	+[b]	22 ± 6	-

[a] Blocked binding of rgp120 MN to CD4.

[b] Blocked binding of rgp120 IIIb, not rgp120 MN, to CD4.

[c] Mean of four determinations calculated using the method of Scatchard (40).

[d] Neutralization of HIV-1_{MN} infectivity *in vitro*.

[e] Anti-rgp120 MN antibody.

[f] Did not neutralize HIV-1 infectivity.

[g] Anti-rgp120 IIIb antibody.

However, when K at position 432 was replaced with A (MN432.A), the binding of all of the CD4 blocking antibodies was markedly reduced (Table 5). Interestingly, the binding of monoclonal antibody 1024 appeared less affected by this substitution than the other monoclonal antibodies (Table 5). Thus, these studies demonstrated that K_{429} and K_{432} were critical for the binding of all of the CD4 blocking monoclonal antibodies, and that K_{419}, K_{421}, and K_{440} did not appear to play a role in monoclonal antibody binding.

Amino acids recognized monoclonal antibodies that block binding of IIIB-rgp120 to CD4. The identification of residues 429 and 432 as being part of the epitope recognized by the MN-rgp120 specific CD4 blocking monoclonal antibodies was particularly interesting since this region was previously found to be implicated in the binding of the 5C2 monoclonal antibody (21). The properties of the 1024 like-monoclonal antibodies and the 5C2 monoclonal antibody differed from the C4 reactive monoclonal antibodies described by other investigators (4, 43) in that the former appeared strain specific and the latter were broadly cross reactive. To account for the strain specificity of these monoclonal antibodies, the sequence of the eleven amino acid peptide of IIIB-rgp120 recognized by monoclonal antibody 5C2 was compared to the corresponding sequence of MN-rgp120. It was found that the IIIB protein differed from the MNB protein at positions 429 where K replaced E and at position 423 where I replaced F (Figure 5). Because it was known from previous studies (33) that the 5C2 monoclonal antibody was unable to bind to gp120 from two strains (i.e., NY-5 and JRcsf) that also possessed E at position 423, it seemed unlikely that this position could account for the strain specificity of

5C2. Sequence comparison (Figure 5) also showed that gp120 from HIV-1_{MB} was unique in that a phenylalanine residue occurred at position 423 whereas the other six strains examined possess an I at this position.

To determine whether residues 423 and/or 429 could account for the type specificity of the 5C2 monoclonal antibody, a mutant of MN-rgp120 was constructed which incorporated an F for I replacement at position 423 (MN.423F). In addition, the MN-rgp120 mutant, MN.429E (described above) was further mutagenized to incorporate a F for I substitution at position 423 (MN.423F), thus resulting in a double mutant (MN.423F,429E) whose sequence was identical to that of IIIB-rgp120 within the 10 amino acid 5C2 epitope (Figure 4). The expression of these mutants in 293s cells was verified by radioimmunoprecipitation using rabbit polyclonal antisera to MN-rgp120. When the binding of the 13H8 monoclonal antibody to a set of mutants incorporating substitutions at position 423 and 429 was examined, it was found that none of the replacements effected the binding of this antibody (data not shown). When the 5C2 monoclonal antibody was examined, it was found that the F for I replacement (MN.423 F) conferred partial reactivity (Table 5). When the double mutant (MN.423F,429E), containing the F for I substitution as well as the E for K substitution was tested, binding that was indistinguishable from that to IIIB-rgp120 was observed (Table 5). These results demonstrated that F at position 423 and E at position 429 both play a role in binding of the 5C2 monoclonal antibody, and suggest that the strain specificity of 5C2 can be attributed to the residues at these positions.

Examination of the sequences of gp120 from the various clones of LAI that have been analyzed revealed that several substrains of LAI differed from each other

in the C4 domain. Thus the sequences of the IIIB (30), Bru (46), and HXB3 (6) clones of LAI were identical at positions 423 and 429 where F and E residues occurred respectively. However, the sequence of the HXB2 substrain (36) differed from the others at these positions where, like MN-rgp120, K replaced E and at position 423 where I replaced F (Figure 5). Similarly, the HX10 and BH10 substrains (36, 37) differed only at position 423 where, like HIV-1_{MN}, I replaced F. Based on the mutagenesis experiments above, it would be predicted that monoclonal antibody 1024 should be able to bind to gp120 from the HXB2 substrain of LAI, but not the HXB3 substrain. If I_{423} was important for binding, then 1024 should also bind the HX10 substrain.

To test this hypothesis, the binding of monoclonal antibody 1024 to the surface cells infected with either IIIB, HXB2, HXB3, and HX10 substrains of HIV-1_{LAI} was measured by flow cytometry. It was found that monoclonal antibody 1024 was able to bind only HXB2 providing further confirmation that residues 423 and 429 were important for the binding of this antibody. The fact that monoclonal antibody 1024 did not bind to HX10 infected cells suggested that I_{423} was not important for the binding of this monoclonal antibody. Thus these studies demonstrate that reactivity with the 1024 monoclonal antibody segregates with the occurrence of F and E residues at positions 423 and 429, respectively, and shows that substrains of HIV-1_{LAI} differ from one another at a functionally significant epitope in the C4 domain.

Neutralizing activity of CD4 blocking antibodies correlates with their binding affinity. To account for the difference in virus neutralizing activity between the CD4 blocking monoclonal antibodies, their gp120 binding affinities were determined by competitive

binding of [^{125}I]-labeled monoclonal antibody to rgp120 (Table 6). Typical Scatchard analysis of data from these assays is shown in Figure 7 (A to C). Linear, one-site binding kinetics were observed for all the monoclonal antibodies to MN-rgp120, suggesting that only a single class of sites was recognized, and that there was no cooperativity between two combining sites of each immunoglobulin molecule. It was found (Figure 7A, Table 6) that monoclonal antibody 1024, which exhibited the most potent virus neutralizing activity (IC_{50} of 0.08 μg per ml), possessed the lowest K_d (2.7 nM). In contrast (Figure 7C, Table 6), monoclonal antibody 1112, the antibody that exhibited the weakest virus neutralizing activity (IC_{50} of 30 μg per ml) possessed the highest K_d (20 nM). K_ds for six additional CD4-blocking monoclonal antibodies raised against MN-rgp120 were also determined (Table 6). It was found that monoclonal antibodies that possessed intermediate K_ds similarly possessed intermediate neutralization IC_{50} values. To explore the relationship between virus neutralizing activity and gp120 binding affinity, the data in Table 6 was plotted in several different ways. It was found that when the K_d of the monoclonal antibodies was plotted as a function of the log of the IC_{50}, a linear relationship was obtained (Figure 8). Using this analysis a correlation coefficient (r) of 0.97) was obtained. Thus, this graph demonstrates that the virus neutralizing activity of these monoclonal antibodies is directly proportional to the gp120 binding affinity, and that the threshold for neutralization at this epitope is defined by the slope of the graph in Figure 8.

A similar analysis was performed with the non-neutralizing CD4 blocking monoclonal antibodies to IIIB-rgp120, 5C2 and 13H8. The binding curve for 13H8 (Figure 7C) showed that it bound to a single class of

sites on IIIB-rgp120 with a K_d of 22 nM. The affinity of 5C2 could not be determined by this assay because at antibody concentrations greater than 5 nM, non-linear (reduced gp120 binding) was observed. This effect was suggestive steric hindrance at these concentrations or negative cooperativity between combining sites. The binding affinity was also determined for the non-neutralizing, non-CD4 blocking monoclonal antibody to MN-rgp120, 1086. The fact that this antibody exhibited a binding affinity similar (9.7 nM) to many of the neutralizing monoclonal antibodies but failed to inhibit infectivity, proves that high antibody binding affinity alone is not sufficient for neutralization.

Effect of C4 Domain Mutants on CD4 binding. Finally, the CD4 binding properties of the series of MN-rgp120 mutants, constructed to localize the C4 domain epitopes, were measured in a qualitative co-immunoprecipitation assay. In these studies the ability of the mutagenized MN-rgp120 variants to co-immunoprecipitate CD4 was evaluated as described previously (21) in a qualitative co-immunoprecipitation assay similar to that described previously (19). Briefly, 293 cells, transfected with plasmids directing the expression of MN-rgp120 variants described in Figure 5, were metabolically labeled with [^{35}S]-methionine, and the growth conditioned cell culture supernatants were incubated with rsCD4. The resulting rsCD4:gp120 complexes were then immunoprecipitated by addition of the CD4 specific monoclonal antibody, 465 (A) or a positive control monoclonal antibody (1034) directed to the V3 domain of MN-rgp120 (B). The immunoprecipitated proteins were resolved by SDS-PAGE and visualized by autoradiography as described previously (3). The samples were: Lane 1, MN.419A; lane 2, MN.421A; lane 3, MN.429E; lane 4,

MN.429A; lane 5, MN.432A; lane 6, MN.440A; lane 7, MN-rgp120. The gel show d that the mutants that block antibody binding do not block binding of CD4. Therefore, the antibodies do not bind to the gp120 CD4-binding contact residues. This indicates that steric hinderance may inhibit antibody binding, rather than that the antibodies bind directly to the CD4 contact residues to inhibit binding.

It was found that all of the variants in which apolar A residue was substituted for the charged K or E residues (e.g., MN.419A, MN.421A, MN.432A, and MN.440A) were still able to co-immunoprecipitate rsCD4. Similarly, the replacement of E for K at position 429 (MN.429E), the replacement of F for I at position 423 (MN.423F) or the mutant which incorporated both mutation (MN.423F,429E) also showed no reduction in their ability to co-immunoprecipitate rsCD4. Thus, radical amino acid substitutions at five positions failed to affect the binding of gp120 to CD4. These results were consistent with previous studies (5, 21, 34) where it was found that only a few of the many mutations that have been induced in this region effected CD4 binding.

This study indicates that neutralizing epitopes in the C4 domain have now been found to be located between about residues 420 and 440. In addition, the critical residues for antibody binding are residues 429 and 432.

EXAMPLE 2

Identification of V2 Neutralizing Epitopes

The procedures described in Example 1 were used to map epitopes in the V2 region of gp120. Table 7 illustrates the results of mutagenicity studies to map V2 neutralizing epitopes. In the table, the columns indicate the comparison of binding of the monoclonal antibodies with wild type (WT) gp120 in comparison to

various mutations of gp120 using standard notation. For example, "G171R" indicates that the glycine (G) at residue 171 has been replaced by an arginine (R). "172A/173A" indicates that the residues at 172 and 173 have been replaced by alanine. The neutralizing monoclonal antibodies tested (MAbs) are listed in the rows. The numerical values in the table are the optical density value of an ELISA assay performed as described in Example 1 to measure the amount of antibody binding. The underlined values indicate significantly reduced binding, indicating the substituted residue is critical for binding of the antibody.

TABLE 7

MAbs	WT	G171R, M174V	172A/ 173A	E187V	187V/188S
6E10	1.00	0.10	1.28	0.60	0.25
1017	1.00	0.70	1.10	0.87	0.04
1022	1.00	0.80	1.10	1.00	0.00
1028	1.00	0.90	1.18	1.07	0.04
1029	1.00	0.83	1.16	1.01	0.16
1019	1.00	0.13	1.30	0.75	0.74
1027	1.00	0.00	1.20	0.80	0.64
1025	1.00	0.69	0.00	0.00	0.83
1088	1.00	0.73	1.12	0.94	0.03
13H8	1.00	0.77	0.78	0.48	0.65

TABLE 7 (continued)

	WT	177A	172A/173A	188A	183A
MAbs					
6E10	1.00	0.36	0.52	0.64	0.43
1017	1.00	0.77	0.77	0.76	0.11
1022	1.00	0.86	0.72	0.14	0.00
1028	1.00	0.93	0.78	0.49	0.04
1029	1.00	0.88	0.85	0.53	0.16
1019	1.00	0.16	0.00	0.41	0.44
1027	1.00	0.00	0.02	0.41	0.49
1025	1.00	0.75	0.0	0.83	0.72
1088	1.00	0.77	0.77	0.53	0.00
13H8	1.00	0.72	0.72	0.53	0.60

As illustrated in Table 7, the study demonstrated that there are a series of overlapping neutralizing epitopes from been found to be located in the V2 region (residues 163 through 200), with most of the epitopes located between residues 163 and 200. In addition, the study indicates that the critical residues in the V2 domain for antibody binding are residues 171, 173, 174, 177, 181, 183, 187, and 188.

EXAMPLE 3

Immunization Studies

gp120 from the MN, GNE_8, and GNE_{16} strains of HIV was prepared by amplifying the gene from each isolate and cloning and expressing the gene in CHO cells as described in Berman et al., *J. Virol.* **66**:4464-4469 (1992). Briefly, the gp160 gene was amplified with two r unds of amplification using the following nested

primers according to the protocol by Kellog et al., pp 337-347 in *PCR Protocols: a guide to m thods and amplification*. Innis et al. (eds.) Academic Press, Inc., New York.

First round primers:

AATAATAGCAATAGTTGTGTGGWCC (W is A or T)

ATTCTTTCCCTTAYAGTAGGCCATCC (Y is T or C)

Second round primers:

GGGAATTCGGATCCAGAGCAGAAGACAGTGGCAATGA

GTCAAGAATTCTTATAGCAAAGCCCTTTCCAA

The primers are SEQ. ID. NOs. 31-34. Each gene is then digested with the restriction endonucleases KpnI and AccI. The resulting fragment was subcloned into the Bluescript (+) phagemid M13 vector (Stratagene, Inc.) and sequenced by the dideoxynucleotide method (Sanger et al., *Proc. Natl. Acad. Sci. USA* 74:5463-5467 (1977)).

A fragment of the gp120 coding region was then used to construct a chimeric gene for expression in mammalian cells, as described in Lasky et al., *Science* 223:209-212 (1986). The 5' end was fused to a polylinker adjacent to a simian virus 40 (SV40) promoter and the 3' end was fused to a polylinker adjacent to the 3' untranslated sequences containing an SV40 polyadenylation signal. The expression vector (MN-rgp120) was co-transfected in CHO cells deficient in production of the enzyme dihydrofolate reductase, along with a plasmid (pSVdhfr) containing a cDNA encoding the selectable marker, dihydrofolate reductase. Cell lines expressing MN-rgp120 were isolated as described in Lasky et al., *Science* 223:209-212 (1986). The recombinant glycoprotein was purified from growth-conditioned cell culture medium by immunoaffinity and ion exchange chromatography as described in Leonard et al., *J. Biol. Chem.* 265:10373-10382 (1990).

gp120 from the GNE_8 and GNE_{16} strains of HIV is prepared in the same manner as described for the MN isolate.

MN-rgp120 (300 μg/injection), GNE_8-rgp120 (300 μg/injection), and GNE_{16}-rgp120 (300 μg/injection) are prepared in an aluminum hydroxide adjuvant (as described in Cordonnier et al., *Nature* **340**:571-574 (1989)). Six chimpanzees are injected at 0, 4, and 32 weeks. Sera are collected and assayed for neutralizing antibody to each strain of HIV at the time of each immunization and three weeks thereafter. At 35 weeks, each of the chimpanzees has significant levels of neutralizing antibodies to each strain.

At 35 weeks, the chimpanzees are randomly assigned to three groups. Each group is challenged with about 10 50% chimpanzee-infectious doses (CID_{50}) each of one of the vaccine isolates. One unimmunized chimpanzee (control) is also injected with the same amount of virus as the immunized chimpanzees for each vaccine strain.

Sera are drawn every two weeks throughout the study and assayed for antibodies to HIV core proteins and for the presence of HIV by PCR amplification and co-cultivation of peripheral blood mononuclear cells (PBMCs) from the chimpanzee together with activated human or chimpanzee PBMCs. The presence of antibodies to core proteins indicates the presence of viral infection as does the detection of amplified viral DNA or viral infection of co-cultivated cells.

The presence of virus is detected by PCR and co-cultivation methods in each unimmunized control animal between weeks 2 and 4 post challenge. Antibodies to core proteins appear in the control chimpanzees at six weeks post challenge. Neither virus nor antibodies are at detectable levels in any of the immunized chimpanzees at one year post challenge,

indicating that the vaccine effectively protects the chimpanzees from infection from each of the challenge strains.

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(ii) TITLE OF INVENTION: HIV Envelope Polypeptides

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 511 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Gly Thr Met Leu Leu Gly Leu Leu Met Ile Cys Ser Ala Thr Glu Lys
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Leu Trp Val Thr Val Tyr Tyr Gly Val Pro Val Trp Lys Glu Ala Thr
35 40 45

Thr Thr Leu Phe Cys Ala Ser Asp Ala Lys Ala Tyr Asp Thr Glu Ala
50 55 60

His Asn Val Trp Ala Thr His Ala Cys Val Pro Thr Asp Pro Asn Pro
65 70 75 80

Gln Glu Val Glu Leu Val Asn Val Thr Glu Asn Phe Asn Met Trp Lys
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Asn Asn Met Val Glu Gln Met His Glu Asp Ile Ile Ser Leu Trp Asp
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Gln Ser Leu Lys Pro Cys Val Lys Leu Thr Pro Leu Cys Val Thr Leu
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Asn Cys Thr Asp Leu Arg Asn Thr Thr Asn Thr Asn Asn Ser Thr Asp
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Asn Asn Asn Ser Lys Ser Glu Gly Thr Ile Lys Gly Gly Glu Met Lys
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180 185 190

Ser Thr Ser Tyr Arg Leu Ile Ser Cys Asn Thr Ser Val Ile Thr Gln
195 200 205

Ala Cys Pro Lys Ile Ser Phe Glu Pro Ile Pro Ile His Tyr Cys Ala
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Pro Ala Gly Phe Ala Ile Leu Lys Cys Asn Asp Lys Lys Phe Ser Gly
225 230 235 240

Lys Gly Ser Cys Lys Asn Val Ser Thr Val Gln Cys Thr His Gly Ile
245 250 255

Arg Pro Val Val Ser Thr Gln Leu Leu Leu Asn Gly Ser Leu Ala Glu
260 265 270

Glu Glu Val Val Ile Arg Ser Glu Asp Phe Thr Asp Asn Ala Lys Thr

275 280 285

Ile Ile Val His Leu Lys Glu Ser Val Gln Ile Asn Cys Thr Arg Pro
290 295 300

Asn Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe
305 310 315 320

Tyr Thr Thr Lys Asn Ile Lys Gly Thr Ile Arg Gln Ala His Cys Ile
325 330 335

Ile Ser Arg Ala Lys Trp Asn Asp Thr Leu Arg Gln Ile Val Ser Lys
340 345 350

Leu Lys Glu Gln Phe Lys Asn Lys Thr Ile Val Phe Asn Pro Ser Ser
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Gly Gly Asp Pro Glu Ile Val Met His Ser Phe Asn Cys Gly Gly Glu
370 375 380

Phe Phe Tyr Cys Asn Thr Ser Pro Leu Phe Asn Ser Ile Trp Asn Gly
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Asn Asn Thr Trp Asn Asn Thr Thr Gly Ser Asn Asn Asn Ile Thr Leu
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450 455 460

Asn Asp Thr Glu Ile Phe Arg Pro Gly Gly Gly Asp Met Arg Asp Asn
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 501 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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35 40 45

Ala Tyr Asp Thr Glu Ala His Asn Val Trp Ala Thr His Ala Cys Val
50 55 60

Pro Thr Asp Pro Asn Pro Gln Glu Val Glu Leu Val Asn Val Thr Glu
65 70 75 80

Asn Phe Asn Met Trp Lys Asn Asn Met Val Glu Gln Met His Glu Asp
85 90 95

Ile Ile Ser Leu Trp Asp Gln Ser Leu Lys Pro Cys Val Lys Leu Thr
100 105 110

Pro Leu Cys Val Thr Leu Asn Cys Thr Asp Leu Arg Asn Thr Thr Asn
115 120 125

Thr Asn Asn Ser Thr Asp Asn Asn Asn Ser Lys Ser Glu Gly Thr Ile
130 135 140

Lys Gly Gly Glu Met Lys Asn Cys Ser Phe Asn Ile Thr Thr Ser Ile
145 150 155 160

Gly Asp Lys Met Gln Lys Glu Tyr Ala Leu Leu Tyr Lys Leu Asp Ile
165 170 175

Glu Pro Ile Asp Asn Asp Ser Thr Ser Tyr Arg Leu Ile Ser Cys Asn
180 185 190

Thr Ser Val Ile Thr Gln Ala Cys Pro Lys Ile Ser Phe Glu Pro Ile
195 200 205

Pro Ile His Tyr Cys Ala Pro Ala Gly Phe Ala Ile Leu Lys Cys Asn
210 215 220

Asp Lys Lys Phe Ser Gly Lys Gly Ser Cys Lys Asn Val Ser Thr Val
225 230 235 240

Gln Cys Thr His Gly Ile Arg Pro Val Val Ser Thr Gln Leu Leu Leu
245 250 255

Asn Gly Ser Leu Ala Glu Glu Glu Val Val Ile Arg Ser Glu Asp Phe
260 265 270

Thr Asp Asn Ala Lys Thr Ile Ile Val His Leu Lys Glu Ser Val Gln
275 280 285

Ile Asn Cys Thr Arg Pro Asn Tyr Asn Lys Arg Lys Arg Ile His Ile
290 295 300

Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys Asn Ile Lys Gly Thr Ile
305 310 315 320

Arg Gln Ala His Cys Ile Ile Ser Arg Ala Lys Trp Asn Asp Thr Leu
325 330 335

Arg Gln Ile Val Ser Lys Leu Lys Glu Gln Phe Lys Asn Lys Thr Ile
340 345 350

Val Phe Asn Pro Ser Ser Gly Gly Asp Pro Glu Ile Val Met His Ser
355 360 365

Phe Asn Cys Gly Gly Glu Phe Phe Tyr Cys Asn Thr Ser Pro Leu Phe
370 375 380

Asn Ser Ile Trp Asn Gly Asn Asn Thr Trp Asn Asn Thr Thr Gly Ser
385 390 395 400

Asn Asn Asn Ile Thr Leu Gln Cys Lys Ile Lys Gln Ile Ile Asn Met
405 410 415

Trp Gln Lys Val Gly Lys Ala Met Tyr Ala Pro Pro Ile Glu Gly Gln
420 425 430

Ile Arg Cys Ser Ser Asn Ile Thr Gly Leu Leu Leu Thr Arg Asp Gly
435 440 445

Gly Glu Asp Thr Asp Thr Asn Asp Thr Glu Ile Phe Arg Pro Gly Gly
450 455 460

Gly Asp Met Arg Asp Asn Trp Arg Ser Glu Leu Tyr Lys Tyr Lys Val
465 470 475 480

Val Thr Ile Glu Pro Leu Gly Val Ala Pro Thr Lys Ala Lys Arg Arg
485 490 495

Val Val Gln Arg Glu
500

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Cys Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Lys Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Cys Arg Ile Lys Gln Phe Ile Asn Met Trp Gln Glu Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Ser Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Cys Arg Ile Lys Gln Ile Ile Asn Met Trp Gln Glu Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Lys Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Cys Arg Ile Lys Gln Ile Ile Asn Met Trp Gln Gly Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Asn Cys
20 25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Cys Arg Ile Lys Gln Ile Ile Asn Arg Trp Gln Glu Val Gly Lys Ala
1 5 10 15

Ile Tyr Ala Pro Pro Ile Ser Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Cys Arg Ile Lys Gln Ile Val Asn Met Trp Gln Arg Val Gly Gln Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Lys Gly Val Ile Lys Cys
20 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Cys Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Gly Ala Gly Gln Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Ser Gly Thr Ile Asn Cys
20 25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Cys Arg Ile Lys Gln Phe Ile Asn Met Trp Gln Glu Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Ser Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Cys Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Lys Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Ser Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Cys Arg Ile Lys Gln Ile Ile Asn Met Trp Gln Glu Val Gly Lys Ala

1 5 10 15

Met Tyr Ala Pro Pro Ile Ser Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Cys Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Glu Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ser Gly Gly Asp Pro Glu Ile Val Met His Ser Phe Asn Cys Gly Gly
1 5 10 15

Glu Phe Phe Tyr Cys Asn Thr Ser Pro Leu Phe Asn Ser Ile Trp Asn
20 25 30

Gly Asn Asn Thr Trp Asn Asn Thr Thr Gly Ser Asn Asn Asn Ile Thr
35 40 45

Leu Gln Cys Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Lys Val Gly
50 55 60

Lys Ala Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys Ser Ser
65 70 75 80

Asn Ile Thr Gly Leu Leu Leu Thr Arg Asp Gly Gly
85 90

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Cys Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Glu Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Cys Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Ala Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Cys Ala Ile Lys Gln Ile Ile Asn Met Trp Gln Lys Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Cys Lys Ile Ala Gln Ile Ile Asn Met Trp Gln Lys Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Cys Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Lys Val Gly Ala Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Cys Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Lys Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Ala Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Cys Arg Ile Lys Gln Phe Ile Asn Met Trp Gln Glu Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Ser Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Cys Lys Ile Lys Gln Phe Ile Asn Met Trp Gln Lys Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Cys Lys Ile Lys Gln Phe Ile Asn Met Trp Gln Glu Val Gly Lys Ala
1 5 10 15

Met Tyr Ala Pro Pro Ile Glu Gly Gln Ile Arg Cys
20 25

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Phe Ile Asn Met Trp Gln Glu Val Gly Lys Ala Met Tyr Ala Pro Pro
1 5 10 15

Ile Ser

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Trp Gln Glu Val Gly Lys Ala Met Tyr Ala Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Gly Lys Ala Met Tyr Ala Pro Pro Ile Lys Gly Gln Ile Arg
1 5 10

WHAT IS CLAIMED IS:

1. A method for making an HIV gp120 subunit vaccine for a geographic region comprising the steps of:
   a. determining a neutralizing epitope in the V2 or C4 domain of gp120 of HIV isolates from the geographic region;
   b. selecting an HIV strain having gp120 which has a neutralizing epitope in the V2 or C4 domain which is common among isolates in the geographic region; and
   c. making an HIV gp120 subunit vaccine from the selected isolate.
2. The method of Claim 1 wherein the neutralizing epitope is determined by determining the amino acid sequence for at least a portion of the V2 or C4 domain.
3. The method of Claim 2 wherein the amino acid sequence is determined by sequencing DNA encoding at least a portion of the V2 or C4 domain.
4. The method of Claim 3 wherein a plurality of isolates having different amino acid sequences for the V2 and C4 domains are selected.
5. The method of Claim 4 wherein a plurality of isolates having different amino acid sequences for the V3 domain is selected.
6. A method for making an HIV gp120 subunit vaccine for a geographic region comprising the steps of:
   a. determining neutralizing epitopes for the V2, V3, and C4 domains of gp120 from HIV isolates from the geographic region;
   b. selecting at least two HIV isolates having different neutralizing epitopes in the V2, V3, or C4 domain; and
   c. making an HIV gp120 subunit vaccine from the selected isolates.

7. The method of Claim 6 wherein each of the selected isolates have one of the most common neutralizing epitopes.

8. A method for making an HIV gp120 subunit vaccine for a geographic region comprising the steps of:
   a. determining the neutralizing epitopes for HIV isolates and the percentage of HIV infections attributable to each strain present in the region;
   b. selecting at least two HIV strains which have the most common neutralizing epitopes in the V2, V3, and C4 domains in the geographic region; and
   c. making an HIV gp120 subunit vaccine from the selected isolates.

9. The method of Claim 8 wherein the isolates are primary patient isolates.

10. The method of Claim 8 wherein the geographic region is North America and the amino acid sequence of gp120 from the HIV isolates MN and GNE_8 are selected.

11. The method of Claim 10 wherein the GNE_{16} isolate is also selected.

12. A multivalent HIV gp120 subunit vaccine.

13. The vaccine of Claim 12 wherein gp120 present in the vaccine is from at least two HIV isolates which have a different neutralizing epitope in the V2 or C4 domain of gp120.

14. The vaccine of Claim 12 wherein gp120 present in the vaccine is from at least two HIV isolates which have a different neutralizing epitope in the V3 domain of gp120.

15. The vaccine of Claim 12 wherein each isolate has a different common neutralizing epitope for the V2 or C4 domains of gp120.

16. The vaccine of Claim 1 wherein gp120 present in the vaccine is from the MN and GNE_8 strains of HIV.
17. The vaccine of Claim 1 wherein gp120 from the GNE_{16} strain of HIV is also present in the vaccine.
18. A DNA sequence of less than 5 kilobases encoding gp120 from GNE_8 and having the nucleotide sequence illustrated in Table 1.
19. A DNA sequence of less than 5 kilobases encoding gp120 from GNE_{16} and having the nucleotide sequence illustrated in Table 2.
20. An expression construct comprising DNA encoding gp120 selected from the group consisting of GNE_8-gp120 and GNE_{16}-gp120 under the transcriptional and translational control of a heterologous promoter.
21. The expression construct of Claim 20 wherein the promoter is a eukaryotic promoter.
22. The expression construct of Claim 21 wherein the DNA encoding gp120 is joined to a heterologous signal sequence.
23. An isolated GNE_8-gp120 polypeptide having the amino acid sequence illustrated in Table 1.
24. An isolated GNE_{16}-gp120 polypeptide having the amino acid sequence illustrated in Table 2.
25. An improved serotyping method for HIV strains comprising determining the serotypes of the V2, V3, and C4 domains of gp120.
26. A truncated gp120 sequence which sequence is free from the C5 domain.
27. The truncated gp120 sequence of Claim 26 wherein the sequence is additionally free from the carboxy terminus through amino acid residue 453 of the gp120 V5 domain.
28. The truncated gp120 sequence of Claim 27 wherein the sequence is additionally free from the gp120 V5 domain.

29. The truncated gp120 sequence of Claim 26 wherein the sequence is additionally free fr m the gp120 signal sequence.

30. The truncated gp120 sequence of Claim 29 wherein the sequence is additionally free from the carboxy terminus through amino acid residue 111 of the gp120 C1 domain.

31. The truncated gp120 sequence of Claim 29 wherein the sequence is additionally free from the carboxy terminus through amino acid residue 117 of the gp120 C1 domain.

32. The truncated gp120 sequence of Claim 26 wherein the sequence is free from the amino terminus of gp120 through residue 111 of the C1 domain and residue 453 through the carboxy terminus of gp120.

33. The truncated gp120 sequence of Claim 26 wherein the sequence is produced by recombinant engineering.

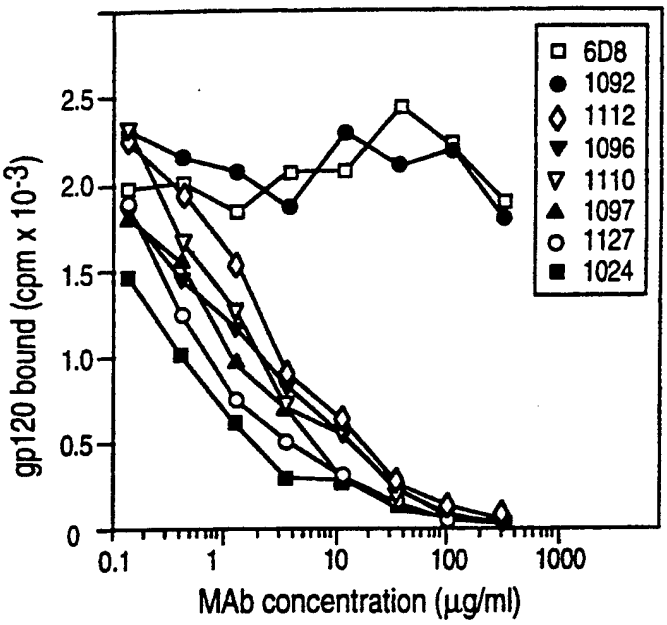
6D8
1092
1112
1096
1110
1097
1127
1024
2.5
2.0
1.5
1.0
0.5
0
0.1
1
10
100
1000
gp120 bound (cpm x 10-3)
MAb concentration (µg/ml)

FIG. 1

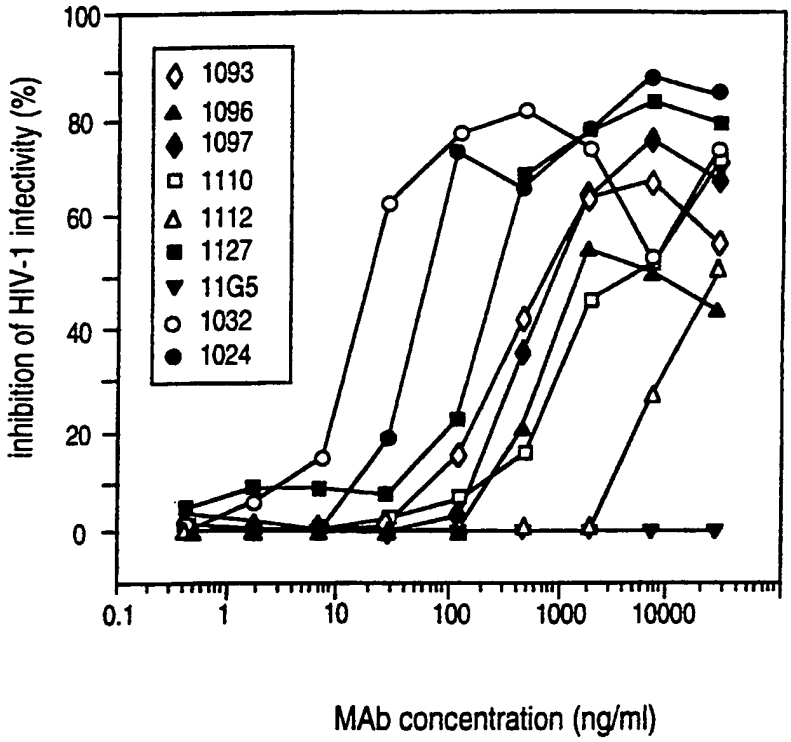
100
80
60
40
20
0
Inhibition of HIV-1 infectivity (%)
1093
1096
1097
1110
1112
1127
11G5
1032
1024
0.1
1
10
100
1000
10000
MAb concentration (ng/ml)

FIG. 2

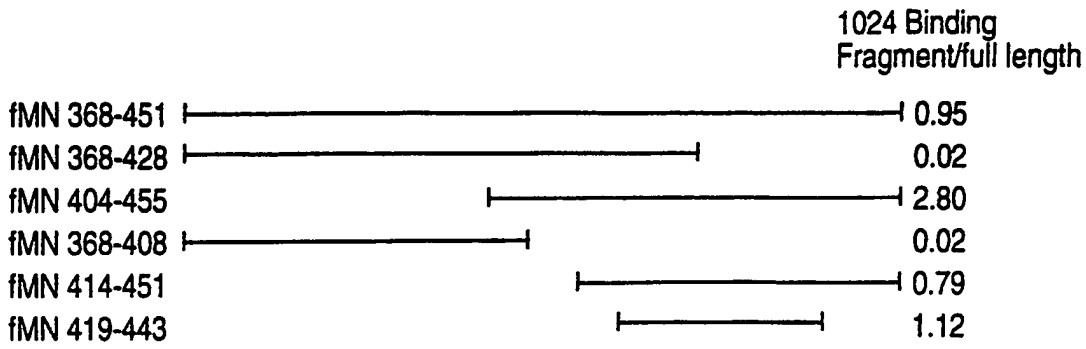
1024 Binding
Fragment/full length
fMN 368-451
0.95
fMN 368-428
0.02
fMN 404-455
2.80
fMN 368-408
0.02
fMN 414-451
0.79
fMN 419-443
1.12

FIG. 3A

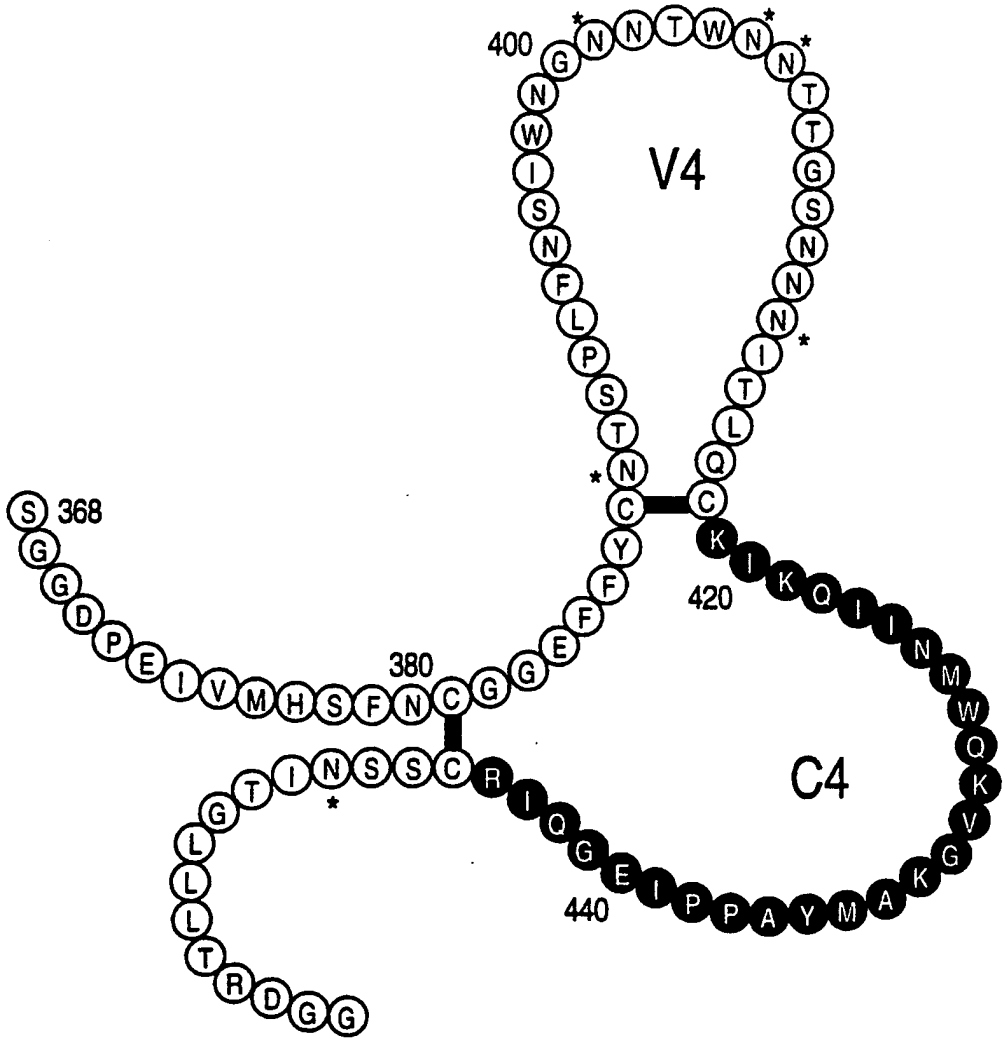
400
V4
368
380
420
440
C4

FIG. 3B

418 445

CKIKQIINMWQKGKAMYAPPIEGQIRC	MN_{GNE}	(SEQ.ID.NO.3)
----------E----------	MN_{1984}	(SEQ.ID.NO.4)
-R---------E----------K-----	JRCSF	(SEQ.ID.NO.5)
-R---------E----------N-	Z6	(SEQ.ID.NO.6)
-R------R--E----I-----S-----	NY5	(SEQ.ID.NO.7)
-R----V----E----------K-V-K-	Z321	(SEQ.ID.NO.8)
----------GA-Q-------S-T-N-	A244	(SEQ.ID.NO.9)
-R---F-----E----------S-----	LAI_{IIIB}, LAI_{BRU}, LAI_{HXB3}	(SEQ.ID.NO.10)
-----I-----K----------S-----	LAI_{HXB2}	(SEQ.ID.NO.11)
-R---I-----E----------S-----	LAI_{BH10}, LAI_{HXB3}	(SEQ.ID.NO.12)
----------E----------	MN_{1984}	(SEQ.ID.NO.13)

FIG. 4

```
418        445
CKIKQIINMWQKVGKAMYAPPIEGQIRC  MN GNE          (SEQ.ID.NO.3)
----------E----------        MN.429E         (SEQ.ID.NO.15)
----------A----------        MN.429A         (SEQ.ID.NO.16)
-A--------------------        MN.419A         (SEQ.ID.NO.17)
---A----------------          MN.421A         (SEQ.ID.NO.18)
----------A----------        MN.432A         (SEQ.ID.NO.19)
----------------A-----        MN.440A         (SEQ.ID.NO.20)
-R---F-----E----------S-----  LAI IIIB        (SEQ.ID.NO.21)
-----F----------             MN.423F         (SEQ.ID.NO.22)
----F-----E----------        MN.423F,429E (SEQ.ID.NO.23)
```

FIG. 5

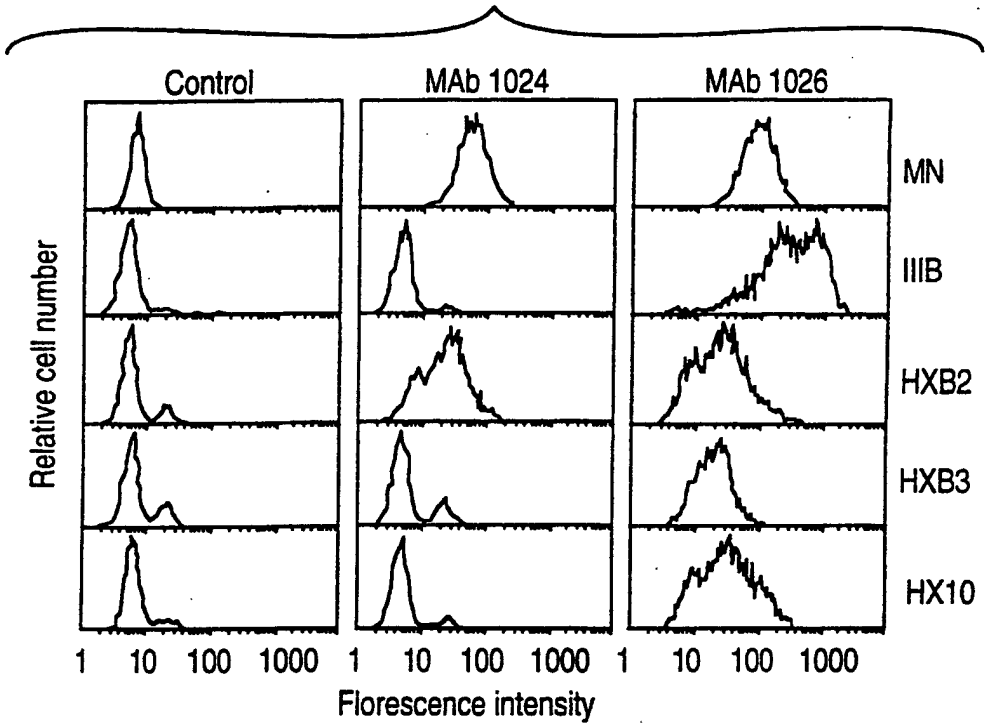
Control
MAb 1024
MAb 1026
MN
IIIB
HXB2
HXB3
HX10
Relative cell number
1 10 100 1000 1 10 100 1000 1 10 100 1000
Florescence intensity

FIG. 6

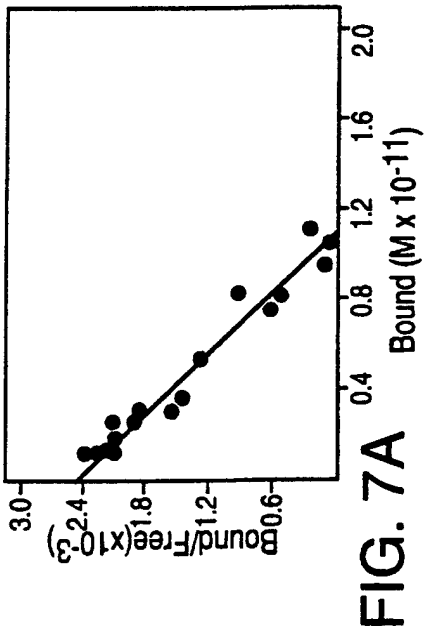

FIG. 7A

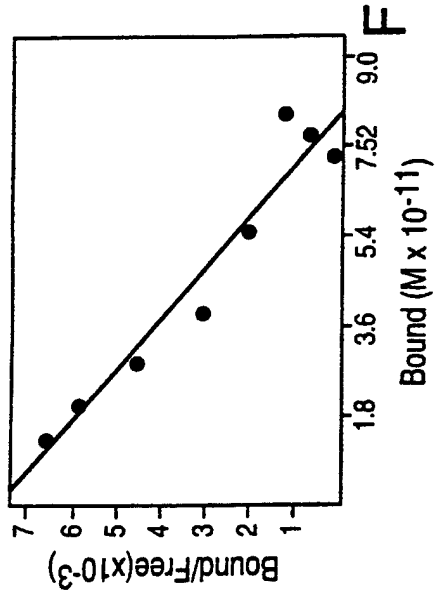

FIG. 7B

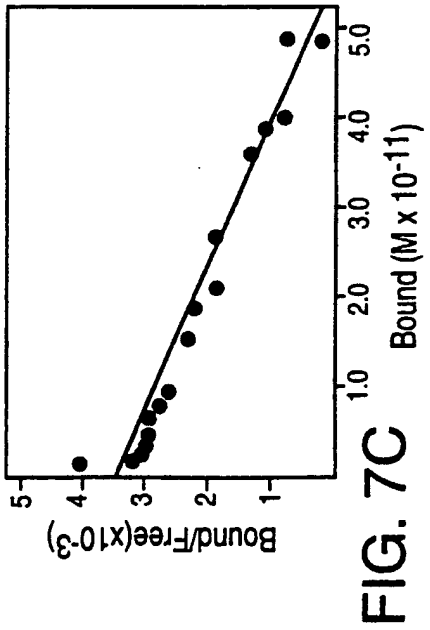

FIG. 7C

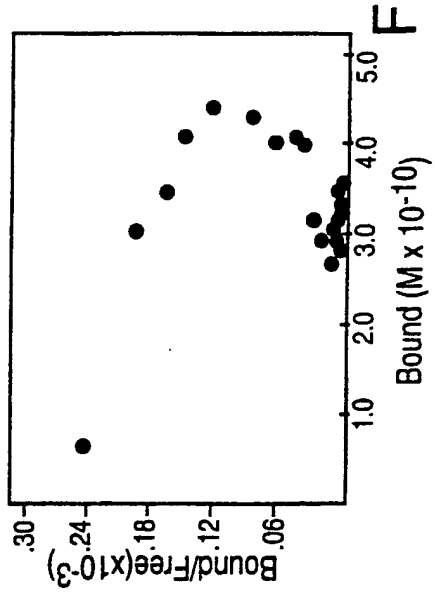

FIG. 7D

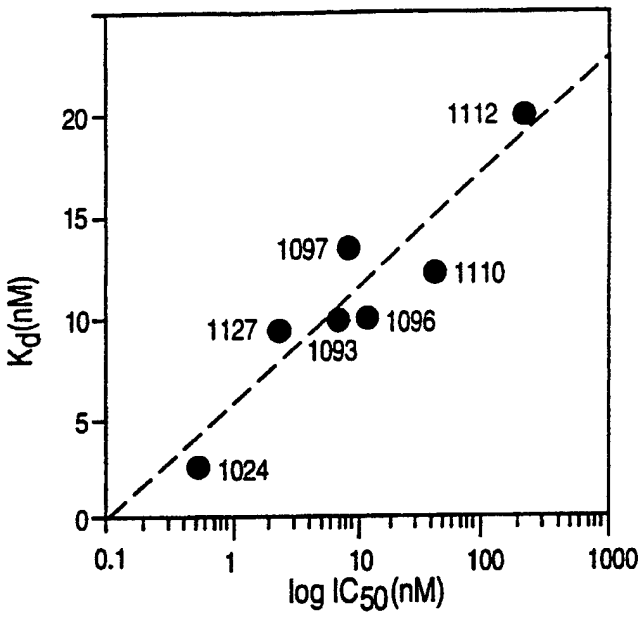
20
15
10
5
0
K_d(nM)
1112
1097
1110
1127
1096
1093
1024
0.1
1
10
100
1000
log IC_{50}(nM)

FIG. 8

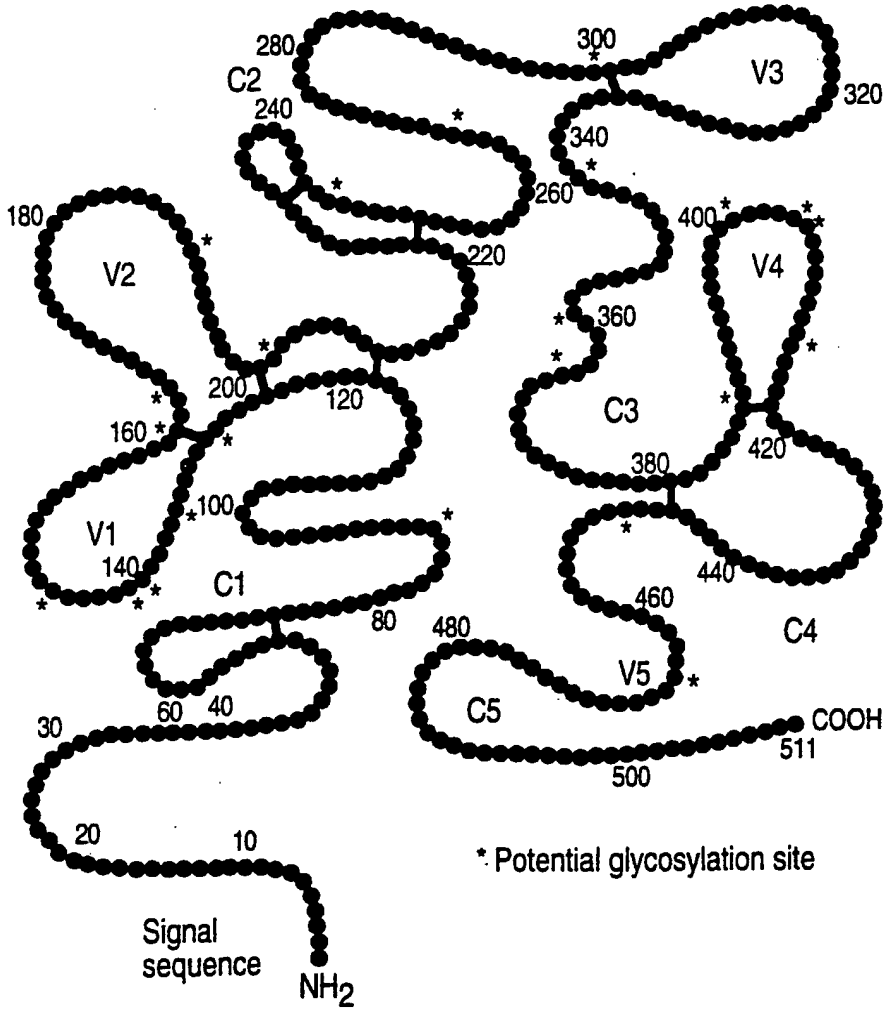
280
300
C2
V3
320
240
340
260
180
400
V2
220
V4
360
200
120
C3
160
420
380
100
V1
140
C1
440
460
80
480
C4
V5
60
40
C5
COOH
30
511
500
20
10
* Potential glycosylation site
Signal
sequence
NH2

FIG. 9

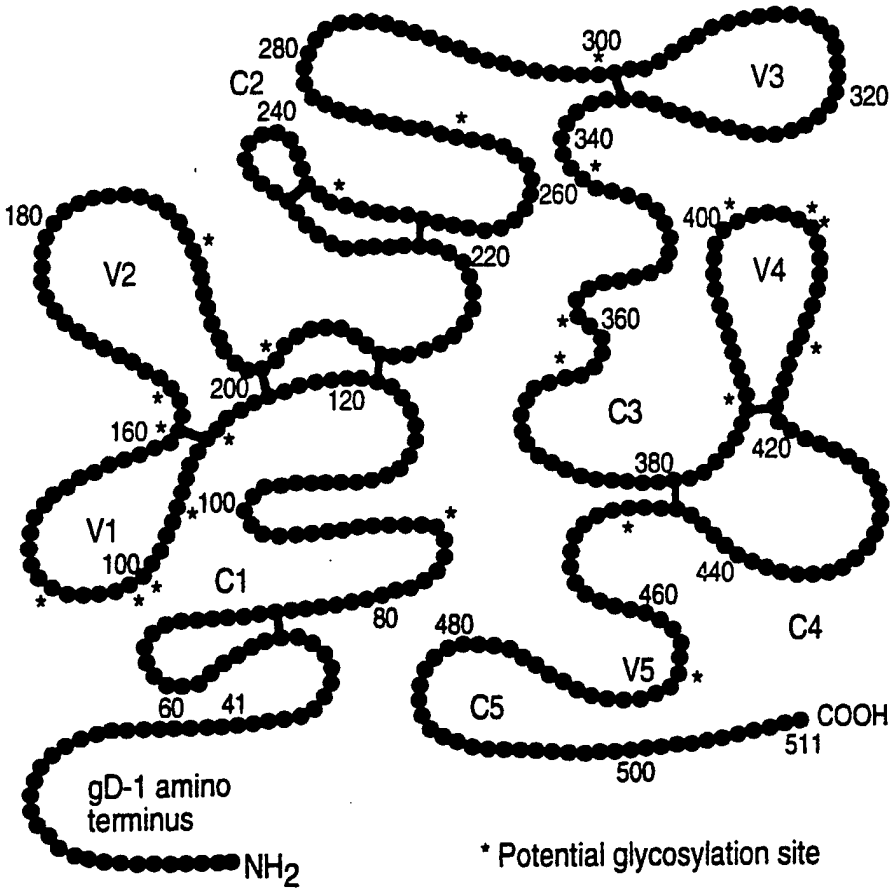
280
300
C2
V3
320
240
340
260
180
400
V2
220
V4
360
200
120
C3
160
420
380
100
V1
100
440
C1
460
80
480
C4
V5
60
41
C5
COOH
511
500
gD-1 amino terminus
NH2
* Potential glycosylation site

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06036

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : IPC(5): A61K 39/12, 37/02; C12N 15/00; C07K 3/00; C07H 15/12
US CL : 424/89; 435/172.1, 320.1; 530/333, 350; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/89; 435/172.1, 320.1; 530/333, 350; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Search terms: HIV, vaccine, variable domains, constant domains, envelope, neutralizing epitopes

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Septieme Colloque Cent Gardes, issued 1992, M. Klein, "Immunogenicity of Synthetic HIV-1 T-B Tandem Epitopes", pages 169-174, see entire article.	5-12, 14, 28-33
Y	Journal of Virology, Vol. 66, No. 9, issued September 1992, M. Thali et al, "Discontinuous, Conserved Neutralization Epitopes Overlapping the CD4-Binding Region of Human Immunodeficiency Virus Type 1 gp120 Envelope Glycoprotein", pages 5635-5641, see entire article.	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
30 SEPTEMBER 1994

Date of mailing of the international search report
20 OCT 1994

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06036

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AIDS Research and Human Retroviruses, Vol. 5, No. 1, issued 1989, "Patterns of Antibody Recognition of Selected Conserved Amino Acid Sequences from the HIV Envelope in Sera from Different Stages of HIV Infection, pages 33-39, see entire article.	1-4
Y	WO, A, 91/ 15512 (GREGORY ET AL.) 17 October 1991, see entire publication.	1-33
Y	Proceedings of the National Academy of Sciences USA, Vol. 89, issued January 1992, Broliden et al, "Identification of Human Neutralization-Inducing Regions of the Human Immunodeficiency Virus Type 1 Envelope Glycoproteins", pages 461-465, see entire article.	5-12, 14, 28-33
Y	Proceedings of the National Academy of Sciences USA, Vol. 89, issued September 1989, Javaherian et al, "Principal Neutralizing Domain of the Human Immunodeficiency Virus Type 1 Envelope Protein", pages 6768-6772, see entire article.	5-12, 14, 28-33